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STUDIES ON ESCHERICHIA COLI HEMOLYSINS AND ANTIHEMOLYSINS

BY

OLOF WIDHOLM

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STUDIES ON ESCHERICHIA COLI HEMOLYSINS AND ANTIHEMOLYSINS

BY

OLOF WIDHOLM

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PREFACE

The subject of this investigation was suggested by my chief, Professor K. O. Renkonen, M. D., head of the Department of Serology and Bacteriology of the University of Helsingfors, to whom I extend sincere thanks for his invaluable guidance in the work and for his personal interest, which have combined to make it a pleasure to carry out the work.

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My thanks are also due to all my colleagues in the Department of Serology and Bacteriology and in the State Serum Institute for ideal working relations.

Mr. F. Ørskov, M. D., of the State Serum Institute, Copenhagen, kindly carried out the typing of the strains of *Esch. coli* for this work, for which I sincerely thank him.

I owe thanks also to Mr. K. Karhunen, Ph. D., who gave me the formula for calculation of the standard error and assisted me in carrying out the computations.

To conclude I wish to thank Miss H.-M. Koskinen, Mag. Phil., for willing assistance in the biochemical work, and Miss S. Strengell for kindly helping me in the practical work in this investigation.

I also thank Miss E. Kaukokallio for translating this report into English.

Financial support for a part of this work has been granted to me by Finska Läkaresällskapet and the University of Helsingfors, to both of which I beg to express my deeply felt thanks.

Helsingfors, February 17, 1953.

Olof Widholm

CONTENTS

Page

INTRODUCTION 7

I. HEMOLYTIC STRAINS OF *ESCH. COLI* 9

A. Incidence of Hemolytic Strains of *Esch. coli* in Various Infections 9

1. Review of the Literature 9

2. Own Investigations 10

B. Distribution of Hemolytic Strains of *Esch. coli* by Serological Groups 11

1. Review of the Literature 11

2. Own Investigations 12

C. Summary 13

II. COLI HEMOLYSINS 14

A. Review of the Literature 14

B. Own Investigations 16

1. Determination of the Potency of Coli Hemolysin 16

2. Tests with Culture Media and the Choice of Peptone Water 18

3. Production of Coli Hemolysin in Oxygen and Nitrogen Atmospheres 19

4. Production of Coli Hemolysin in Casein Hydrolysate 21

5. Production of Coli Hemolysin in Robinson's Medium 23

6. Other Factors Influencing the Production of Coli Hemolysin 26

7. Effect of Temperature on Coli Hemolysin 28

8. Separation of Coli Hemolysin from the Bacterial Cells.. 28

9. Precipitation and Concentration of Coli Hemolysin.... 30

a. Precipitation with Methyl Alcohol at —10° C..... 30

b. Precipitation with Ammonium Sulphate..... 34

10. Lyophilization of Coli Hemolysin Precipitate..... 35

11. Storage of Coli Hemolysin 36

C. Summary 38

III. SEROLOGICAL STUDIES ON ANTICOLILYSINS 40

A. Colilysin and Anticolilysin in Animal Experiments 40

1. Immunization of Rabbits 40

2. Titration of Anticolilysin in Rabbit Sera 41

B. Anticolilysin in »Normal» Human Sera 44

1. Method of Titration 44

2. »Normal» Series 45

3. Mean Error of the Anticolilysin Reaction 47

4. Specificity of the Anticolilysin Reaction 48

C. Anticolilysin in Patient Sera 49

1. Review of the Literature on Incidence of *Esch. coli* in Various Infectious Diseases 49

2. Own Investigations 51

a. Anticolilysin (ACL) in Infections of the Urinary Tract Caused by *Esch. coli* 51

b. ACL in Diseases of the Gallbladder 53

c. ACL in Infectious Gynecological Diseases 55

d. ACL in Infantile Enteritis and Gastro-Enteritis 57

e. ACL in Various Other Diseases 58

D. Summary 59

IV. DISCUSSION 62

V. CONCLUSIONS 69

BIBLIOGRAPHY 72

40
40
40
41
44
44
45
47
48
49
49
51
51
53
55
57
58
59
62
69
72

INTRODUCTION

The great interest felt toward the antistreptolysin, antistaphylolysin and antipneumolysin reactions has given an incentive to the investigation of hemolysin production by hemolytic strains of *Esch. coli* and to attempts to develop, in analogy with the above mentioned reactions, a clinically serviceable anticolilysin reaction.

In 1903, Kayser (13) described a heat-resistant, filtrable hemolysin produced by *Bact. coli*, which gave a highly sensitive reaction with defibrinated dog red cells. However, very little attention was given to the subject until in 1946, in connection with a work on the pathogenicity of *Bact. coli* in relation to the different serological groups, Sjöstedt (29) made an attempt to repeat Kayser's experiments. However, he did not succeed in obtaining results. The efforts of Robinson (24) to produce a filtrable, heat-resistant colihemolysin according to Kayser's method also failed. Robinson reached the conclusion that the hemolysis obtained by Kayser was not produced by a colilysin and that it may be ascribed to the carbonates developed during bacterial growth. On the other hand, Robinson obtained a thermolabile colilysin by using a specially developed culture medium, but he did not succeed in separating the hemolysin from the living bacteria.

In view of the very few and diverging reports in the literature, I have endeavored to study more closely the production and nature of coli hemolysins and to investigate the possibilities of developing a clinically serviceable anticolilysin reaction.

It was therefore the aim of my investigation to cover the following aspects of this subject:

1) The isolation of strongly hemolytic strains of *Esch. coli;*

2) The suitability of different blood cells as indicators of the hemolytic power of *Esch. coli;*

3) The production, stability, concentration and antigenic properties of the hemolysin produced by *Esch. coli;*

4) The anticolilysin reaction and the presence of anticolilysins in the sera of healthy and diseased persons.

CHAPTER I

HEMOLYTIC STRAINS OF *ESCH. COLI*

A. INCIDENCE OF HEMOLYTIC STRAINS OF *ESCH. COLI* IN VARIOUS INFECTIONS

1. REVIEW OF THE LITERATURE

It was demonstrated by Kayser (13) that certain strains of *Bact. coli* have a hemolytic power.

Meyer & Löwenberg (19) found that 25 out of 61 strains of *Bact. coli* isolated from urine were hemolytic, and that 64.0 per cent of the hemolytic strains were from male and 34.6 per cent from female cases. Bitter & Gundel (2) examined 616 samples of urine from colibacillary infections and were able to demonstrate the presence of hemolytic strains in 65 cases. Vahlne (31) found hemolytic activity in 25 per cent of urinary coli strains.

In examining the coli flora of patients with various intestinal diseases, Dudgeon, Wordley & Bawtree (4) found hemolytic strains in 35 per cent of cases, as compared with an incidence of 12 per cent in healthy persons. In a similar examination made by Meyer & Löwenberg (19) the incidence of hemolytic strains in feces in intestinal diseases was reported to be 58 per cent as against 13 per cent in normal persons. Klingenstein (14), in a series of 42 cases of gastro-intestinal disorders. found hemolytic coli strains in the feces in 55 per cent of cases. Vahlne (31) demonstrated hemolytic fecal strains in 9 per cent of healthy persons, whereas the incidence was 19 per cent in peritonitis and 10 per cent in appendicitis.

Some reports in the literature advance the opinion that the hemolytic strains of *Esch. coli* produce a more acute disease condition than the non-hemolytic strains.

In most of the investigations referred to above the hemolytic activity was determined by culture of the bacteria in a peptone-saline medium with the addition of 20 per cent of three times washed sterile horse red cells. The readings were made after 24 hours' growth at + 37° C.

2. OWN INVESTIGATIONS

The strains of *Esch. coli* employed in these experiments were isolated from routine samples obtained from the Department of Serology and Bacteriology of the University of Helsinki, the State Serum Institute, and the State Laboratory of Veterinary Medicine, all in Helsinki.

From a total of 200 cultures of various bacteria there were selected 130 cultures which with fairly great certainty could be regarded to be *Esch. coli*. The determination was made on the basis of the following properties of the bacteria:

Microscopically Gram-negative rods;
Typical colonies on the Conradi-Drigalski plate;
Production of acid from glucose, mannite, lactose and sucrose;
Production of gas from glucose and sucrose;
Positive indole test;
Positive methyl red reaction;
Negative citrate reaction;
Negative Voges-Proskauer test;
Negative urea test.

The above mentioned reactions were carried out according to the methods of Thjøtta (30) and Kauffmann (11).

The determinations of the hemolytic power of the strains were made as follows: Two per cent defibrinated, sterile sheep red cells were added to a tube of 1 per cent peptone water, pH 7.6. The amount of medium in each tube was usually 2 ml. After inoculation of the medium with a loopful of bacterial culture and growth at + 37° C. in the thermostat for 24 hours, the tubes were examined for complete or partial hemolysis. Those strains which in the preliminary test yielded complete hemolysis also produced an active hemolysin in most cases.

Table 1 shows the distribution of the strains according to origin and the number and proportion of hemolytic strains, partial hemolysis included.

TABLE 1

SOURCE OF BACTERIA AND INCIDENCE OF HEMOLYTIC POWER OF 130 STRAINS OF *Esch. coli*

Origin	No. of Strains	Hemolytic Strains	
		No.	%
Feces	64	17	27 %
Urine	22	9	41 %
Pus	4	4	(100 %)
Sputum	3	2	(67 %)
Throat	1	1	(100 %)
Pus from bovine mastitis	36	14	39 %
Total	130	47	36 %

About one-half of the strains were isolated from cases of intestinal disorder, but the incidence of hemolytic strains in these fecal cultures was no greater than 27 per cent. The highest incidence of hemolytic cultures (41 per cent) was seen in those isolated from sterile urine. It deserves to be noted that the samples from bovine mastitis, all of which were septic and many fatal, yielded hemolytic cultures in 39 per cent. This incidence is approximately as high as in the fecal and urinary strains of human origin. No definite conclusions can be drawn with regard to the other groups in view of the small number of cultures obtained, but it is noteworthy that the 4 strains isolated from pus were all hemolytic.

B. DISTRIBUTION OF HEMOLYTIC STRAINS OF *ESCH. COLI* BY SEROLOGICAL GROUPS

1. REVIEW OF THE LITERATURE

Several investigators, *e.g.*, Dudgeon, Wordley & Bawtree (4), Meyer & Löwenberg (19), Klingenstein (14) and Harada (7) have noted a connection between the different serological groups and the hemolytic strains of *Esch. coli*. Kauffmann (10) has demonstrated that the hemolytic strains chiefly belong to O groups 2,

3, 4 and 6. This finding has been corroborated by Knipschildt (15). According to Vahlne (31) the hemolytic power is associated with O groups 1, 2, 4, 6, 8, 9, 18 and 21, among which type 18: 5 alone contained both hemolytic and non-hemolytic strains. Sjöstedt (29) has confirmed Vahlne's findings and states that the hemolytic power is found in O groups 2, 3, 4, 6, and 22 in particular.

Kauffmann & Dupont (12) have shown that strains of *Esch. coli* isolated from infantile diarrhea belong chiefly to O groups 55 and 111.

2. OWN INVESTIGATIONS

Twelve of the strains of *Esch. coli* showing the strongest hemolysis were selected and their typing was carried out at the State Serum Institute, Copenhagen. The results of the typing are shown in Table 2.

TABLE 2

DISTRIBUTION OF HEMOLYTIC STRAINS OF *Esch. coli* BY O GROUPS

Origin and Strain No.	O Group No.								
	2	6	22	33	54	75	76	83	87
Sterile urine:									
284	—	—	—	X	—	—	—	—	X
2798	—	—	—	—	—	X	—	—	—
4658	X	—	—	—	—	—	—	—	—
5329	X	—	—	—	—	—	—	—	—
5445	—	—	—	—	—	X	—	—	—
5472	—	—	X	—	—	—	X	X	—
5533	—	—	X	—	—	—	X	X	—
5598	—	—	—	—	X	—	—	—	—
5631	—	—	—	—	—	X	—	—	—
Sputum:									
2063	—	—	—	—	X	—	—	—	—
5408	X	—	—	—	—	—	—	—	—
Bovine mastitis:									
24	—	X	—	X	—	—	—	—	X

Two of the nine strains isolated from sterile urine belonged to O group 2, and 2 strains gave the reaction of O group 22. None of the other strains from this source were found to be of the types under which the hemolytic strains generally are classified. One

of the 2 hemolytic strains from sputum was of O group 2, and the strain isolated from bovine mastitis gave the reactions of O groups 6, 33 and 87.

All the hemolytic fecal strains mentioned in Section A. were tested at the State Serum Institute, Helsinki, with a view to determining if any of them belonged to O groups 55 or 111. However, the agglutination tests were negative in all cases.

C. SUMMARY

A total of 200 different bacterial cultures were examined and 130 cultures were diagnosed as *Esch. coli*. The hemolytic power of the strains of *Esch. coli* was determined by cultivation in 1 per cent peptone water to which 2 per cent defibrinated, sterile sheep red cells were added.

The origin and incidence of strains giving complete or partial hemolysis was as follows: Feces from intestinal disorders, 27 per cent; sterile urine, 41 per cent; pus, 100 per cent (4 strains only); sputum, 67 per cent (3 strains only); and bovine mastitis, 39 per cent. The only strain isolated from the throat was hemolytic.

Twelve of the strains giving the strongest hemolysis were typed and referred to various O groups.

CHAPTER II

COLI HEMOLYSINS

A. REVIEW OF THE LITERATURE

Kayser (13) separated colilysin from two pyogenic strains of *Bact. coli* isolated from pus. The hemolysin was produced after 4 days' growth in slightly acid meat-peptone water at + 37° C. He found that it was filtrable through a Chamberland filter and that there was no difference in the hemolytic power of the filtrate and that of the fluid containing the bacterium. According to Kayser the decisive factors in the production of coli hemolysin were the degree of acidity of the culture medium, which was only to be mildly acid, and the increasing alkalinity of the medium during the production of lysin. Neutralization of an alkaline coli filtrate by N./10 oxalic acid lowered its activity. Heating of the colilysin at + 120° C. did not weaken the hemolytic power.

Kayser stored the coli filtrate in hermetically sealed, sterile flasks in the refrigerator for 3 months with no change in its activity. The alkaline reaction of the lysin remained constant during this period.

Sjöstedt (29) made an unsuccessful attempt to obtain a filtrable coli hemolysin from *Bact. coli* cultures of varying age grown in different media (semifluid agar, placental broth, beef extract broth, peptone water) with and without glucose. Variation of the pH and reduction with sodium hydrosulphite were equally unsuccessful.

In investigations on the conditions for hemolysis by *Bact. coli* Robinson (24) started out with a culture medium consisting of: ($NaC_2H_3O_2 \cdot 3\ H_2O$), 2.5 g.; ($Na_3C_6H_5O_7 \cdot 5\ H_2O$), 2.5 g.; NaCl, 5.0 g.; KH_2PO_4, 1.0 g.; $MgSO_4 \cdot 7\ H_2O$, 0.1 g.; 1/N.-NaOH,

5.7 ml.; H_2O to make 1 l. After autoclaving, 1 volume of a sterile 10 per cent (w/v) solution of ammonium sulphate was added to 100 volumes of the basal medium.

By growing various hemolytic strains of the bacterium in this medium Robinson endeavored to demonstrate the presence of hemolysin by the addition, either before or after growth, of washed human red cells or defibrinated human blood. No hemolysis occurred, although similar experiments gave rise to hemolysis in peptone water. The addition of 0.25 per cent Bacto-Peptone in the first mentioned medium also failed to give results. He therefore studied the effect of the individual ingredients in blood-peptone water.

As it was found that sodium citrate, even in small amounts, was inhibitory to hemolysis and that potassium oxalate and sodium acetate and lactate had a similar effect, Robinson concluded that the calcium ion plays an important rôle in the hemolysis. Even without inhibitors the amount of calcium in the 1 per cent Bacto-Peptone water was below the optimum, which was attained only by the addition of 0.1 per cent $CaCl_2$.

On the basis of these findings Robinson compounded his final medium consisting of (in percents, w/v): $(NH_4)_2SO_4$, 10; KH_2PO_4, 0.5; NaCl, 5.0; $MgSO_4 \cdot 7\ H_2O$, 0.05; citric acid (5 H_2O), 2.0; lactic acid (Analar), 4.0 v/v. For use, 100 ml. of this solution was diluted to c. 900 ml., then 20 ml. of a 10 per cent $CaCl_2 \cdot 6\ H_2O$ solution and 60 ml. of a 1/N.-NaOH solution were added, the volume was adjusted to 1 l. and the medium autoclaved at +120° C. for 30 min. When cool it was adjusted to pH 5.9 by adding c. 13 ml. of a 1/N.-NaOH solution and re-autoclaved at + 115° C. for 15 min.

Using this medium Robinson was able to obtain good hemolysis with 5 different strains of *Bact. coli* when human blood or washed red cells were added before or after growth. No hemolysis occurred if a 5 per cent solution of $MgSO_4 \cdot 7\ H_2O$ was substituted for the 10 per cent solution of $CaCl_2 \cdot 6\ H_2O$. Robinson was of the opinion that the presence of calcium ions is necessary for the actual hemolytic process but not for the production of the hemolytic suspension.

Robinson's efforts to separate the hemolysin from the living bacteria were unsuccessful. The hemolytic power was destroyed by heating at +60° C. No results were obtained with Seitz

filtration, dialysis through cellophane, extraction of cultures with organic solvents, or by grinding or shaking active bacterial sediments with serum or strong peptone solution. Sodium dithionite, which is used as a reducing agent in the production of streptolysin, also failed to give results. Unsuccessful efforts were also made to produce hemolysis by culturing hemolytic strains in peptone water or Robinson's defined medium in collodion sacs suspended in saline containing blood.

Robinson reported that the maximal hemolytic activity occurs during the logarithmic phase of growth, and that it seems to be produced by one generation of the bacterium which is very strongly hemolytic.

Robinson was able to maintain the hemolytic power of the bacterial suspensions for about 3 weeks by refrigeration at $\pm 0°$ C.

B. OWN INVESTIGATIONS

1. DETERMINATION OF THE POTENCY OF COLI HEMOLYSIN

In the production of colilysin it is very important that the presence and amount of colilysin in the culture medium can be determined.

In these experiments, as in the investigations reported in the literature on other bacterial hemolysins, the lysin concentration is indicated as the minimum hemolytic dose (MHD). This is the smallest amount of hemolysin which, when diluted with physiological saline solution to the volume of 1.0 ml., will give complete or over 50 per cent hemolysis in 0.5 ml. of a red cell suspension.

In order to find the suspension of red cells most suitable for this investigation, a series of tests was carried out using given lysin concentrations and varying suspensions of red cells. Human, sheep, horse, rabbit and guinea pig cells were tested.

The blood was defibrinated by shaking in flasks with glass beads until cool. The red cells were then washed three times by centrifugation in physiological saline, and 1, 2 and 3 per cent suspensions of each type of red cells were prepared in physiological saline. The hemolysin under test was diluted with physiological saline solution to a volume of 1.0 ml. in a falling series of concentrations containing 0.5, 0.25, 0.125, 0.064 . . . 0.004 ml. of lysin.

Three such series of lysin dilutions were prepared for each type of red cells, corresponding to the 1, 2 and 3 per cent cell suspensions. Then 0.5 ml. of a given cell suspension was added into each tube in the corresponding test series and the tubes were shaken and incubated in the water-bath at + 37° C. for 1 hour. After standing for 1 hour at room temperature to allow thorough sedimentation of non-hemolyzed cells, the tubes were examined. The degrees of hemolysis were classified as complete C, nearly complete (C), partial P, slight (P), and no hemolysis O.

TABLE 3

SENSITIVITY OF VARIOUS RED BLOOD CELLS TO COLI HEMOLYSIN

Red Cell Suspension		Hemolysin Concentration							
		0.500	0.250	0.125	0.064	0.032	0.016	0.008	0.004
Human	1%	C	C	C	C	C	C	(P)	O
»	2%	C	C	C	C	C	O	O	O
»	3%	C	C	C	C	C	O	O	O
Sheep	1%	C	C	C	C	C	C	(P)	O
»	2%	C	C	C	C	C	C	(P)	O
»	3%	C	C	C	C	C	P	(P)	O
Horse	1%	C	C	C	C	C	C	O	O
»	2%	C	C	C	C	C	(P)	O	O
»	3%	C	C	C	C	C	(P)	O	O
Rabbit	1%	C	C	C	C	O	O	O	O
»	2%	C	C	C	C	C	O	O	O
»	3%	C	C	C	C	P	O	O	O
Guinea pig	1%	C	C	C	C	C	C	O	O
» »	2%	C	C	C	C	C	(P)	O	O
» »	3%	C	C	C	C	(P)	O	O	O

The results are given in Table 3 and show that sheep red cells were the most sensitive to colilysin. A 2 per cent suspension of these cells appeared to be preferable, since it gave clearly demonstrable results and since fresh sheep red cells were always available at the laboratory. I did not find the horse red cells, which commonly are used for determination of the hemolytic power of *Esch. coli*, equally sensitive. In this investigation, therefore, a 2 per cent suspension of sheep red cells was used in all determinations of the MHD and of the anticolilysin titer.

2. TESTS WITH CULTURE MEDIA AND THE CHOICE OF PEPTONE WATER

For the purpose of obtaining the culture medium most suitable for the development of a serviceable colilysin, I carried out a number of preliminary experiments using as culture medium beef broth compounded in various ways, under varying oxygen conditions. Already at an early stage of the work it became apparent that the lysin in question is extremely labile and difficult to handle.

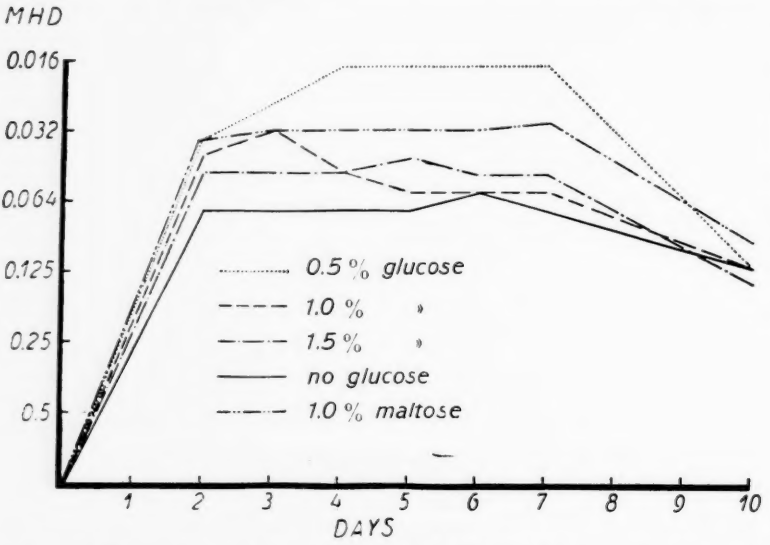

Fig. 1. — Diagram showing production of colilysin in 1 per cent peptone water with addition of different amounts of carbohydrates.

In preliminary tests with the hemolytic strains of *Esch. coli* it was found that lysin was produced in ordinary broth, pH 7.6, inoculated with a hemolytic strain. To obtain a medium of as simple composition as possible, an 1 per cent solution of peptone water was prepared with Witte's peptone, adjusted to pH 7.6 and autoclaved. Then 50 ml. of this medium was inoculated with 1 ml. of a culture of *Esch. coli* strain No. 24 grown in 2 per cent sheep red cell-peptone water at + 37° C. for 16 hours. The inoculated medium in a 100 ml. Erlenmeyer flask was placed in the thermostat at + 37° C. The hemolysis determinations made at intervals of 2 hours showed clearly the production of hemolysin after 2 hours' growth and an increase up to c. 5—6 hours,

after which a decline in the activity set in. After culture for 24 hours the lysin was almost completely inactive.

In an attempt to enhance the activity, varying amounts of carbohydrate were added to the medium. For this purpose 50 per cent sterile solutions of glucose and maltose were used. The glucose was added in amounts of 0.5, 1.0 and 1.5 per cent to three 100 ml. Erlenmeyer flasks, each containing 50 ml. peptone water at pH 7.6. A similar flask containing peptone water only was used as the control. To a fifth flask containing an identical amount of peptone water 1 per cent of maltose in 50 per cent solution was added. All the flasks were inoculated with 1 ml. of a 16 hour culture of strain No. 24 grown in 2 per cent sheep red cell-peptone water. The flasks were placed in the thermostat at + 37° C. and a series of hemolysis tests was carried out at intervals of 2 hours. The results are shown in Fig. 1.

It will be noted that the most active hemolysin was produced in a medium containing 0.5 per cent glucose, and that, on the whole, a more active hemolysin was obtained in media containing carbohydrate than in that containing none. In the presence of 0.5 per cent glucose, strong hemolysis occurred already after 2 hours' growth, with an increase up to 4 hours. After 7 hours' growth a continuous decline in the hemolytic power set in.

3. PRODUCTION OF COLI HEMOLYSIN IN OXYGEN AND NITROGEN ATMOSPHERES

For studying the relationship between the production of lysin and the oxygen supply the bacteria were grown in flasks containing 1 per cent peptone water in the presence of various gases, *i.e.*, O_2, N_2, and $N_2 + O_2$. Three Erlenmeyer flasks of 250 ml. each, containing 100 ml. peptone water and 0.5 per cent glucose, at an initial pH of 7.85, were closed with rubber stoppers through which a capillary tube was passed to the bottom of the flask. A shorter glass tube provided an exit for the gas. A fourth flask used as the control was similar in content but was only closed with a plug of cellulose wadding, and no gas was blown through this medium.

Inoculation of the medium in the flasks was made with 2 ml. of a 16 hour culture of *Esch. coli* strain No. 24 grown in 2 per cent sheep red cell-peptone water. Incubation was carried out in the thermostat at + 37° C. A container with O_2, N_2, or $N_2 + O_2$,

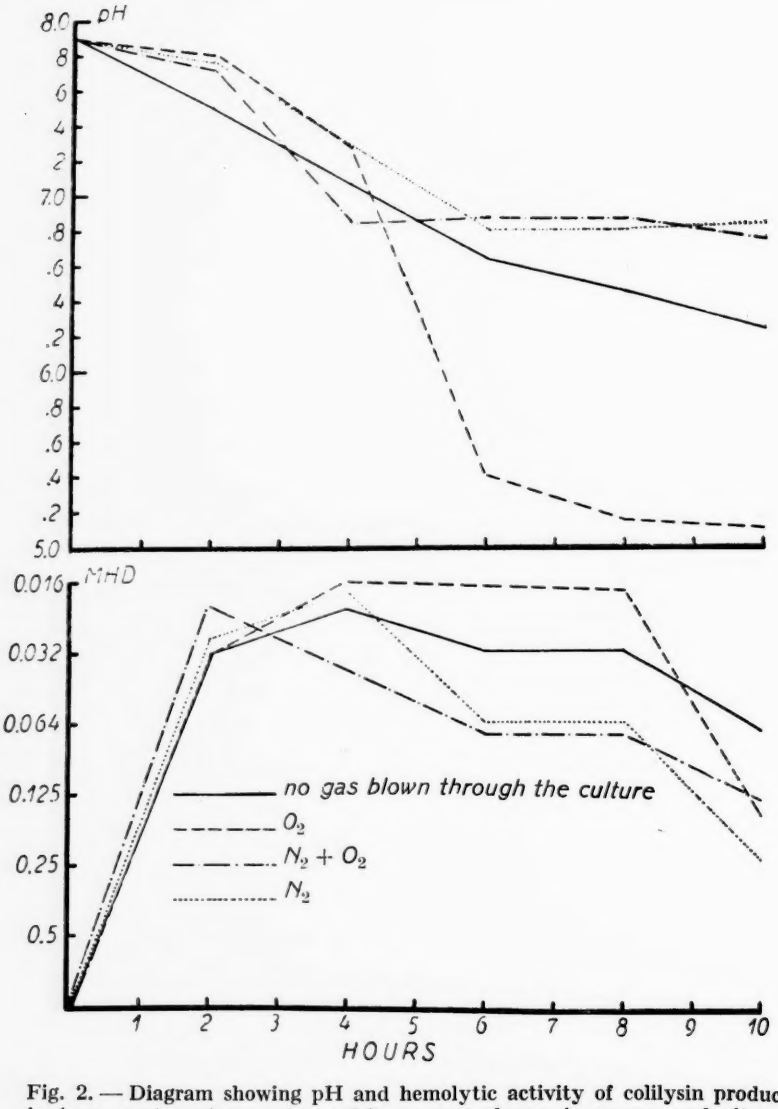

Fig. 2. — Diagram showing pH and hemolytic activity of colilysin produced in 1 per cent peptone water + 0.5 per cent glucose in oxygen and nitrogen atmospheres.

respectively, was attached to each of the three flasks with rubber stoppers. To obtain as oxygen-free nitrogen as possible the gas was passed through a container filled with SiO_2 stones and a glowing copper wire spiral. The rate of gas supply was adjusted to about 25 bubbles per minute. Samples were drawn from the

four flasks by sterile procedure at 2 hour intervals for the determination of hemolysis and pH.

It will be seen from the graph in Fig. 2 on the progress of hemolysis that lysin production in all the flasks had a parallel course during the first 2 hours. The activity of the lysin in the oxygen atmosphere increased thereafter, reached maximum at 4 hours, and then remained stable up to 8 hours. In the 2 flasks supplied with nitrogen, approximately the same maximum was attained but a decrease set in at 2 and 4 hours. Accordingly, the decline in the activity of the hemolysin is not greatly influenced by the presence of either oxygen or nitrogen.

The pH measurements were made by means of a pH meter (Beckmann), which was controlled against a standard solution previous to every measurement. In the graph showing the pH values determined at the time of evaluation of the hemolysis, a parallel decline will be noted in the curves up to 4 hours, after which there was an abrupt decline in the pH of the medium through which oxygen was blown. No definite conclusions on the inactivation of the lysin because of reduced pH levels could be drawn, but a certain degree of correlation seemed to exist between declining pH values and diminishing activity of the colilysin.

4. PRODUCTION OF COLI HEMOLYSIN IN CASEIN HYDROLYSATE

With a view to possible analysis of the nature and composition of the coli hemolysin in a later phase of this work, an attempt was made to produce coli hemolysin in a medium having as well known a protein structure as possible. For this purpose, casein hydrolysate prepared according to the method of Holz (9) and used in the production of diphtheria toxin (Pharmaceutical Factory »Orion») was employed.

As in the preceding experiment, the culture medium was adjusted to pH 7.6, filtered and autoclaved. Two different types of casein hydrolysate — with and without added yeast autolysate — were tested. The amount of yeast autolysate added was 1 ml. of an 1 per cent solution to 1 l. of casein hydrolysate.

For this experiment two 200 ml. Erlenmeyer flasks filled with each medium and closed with plugs of cellulose wadding were used. After the addition of 0.5 per cent glucose in the form of a 50 per

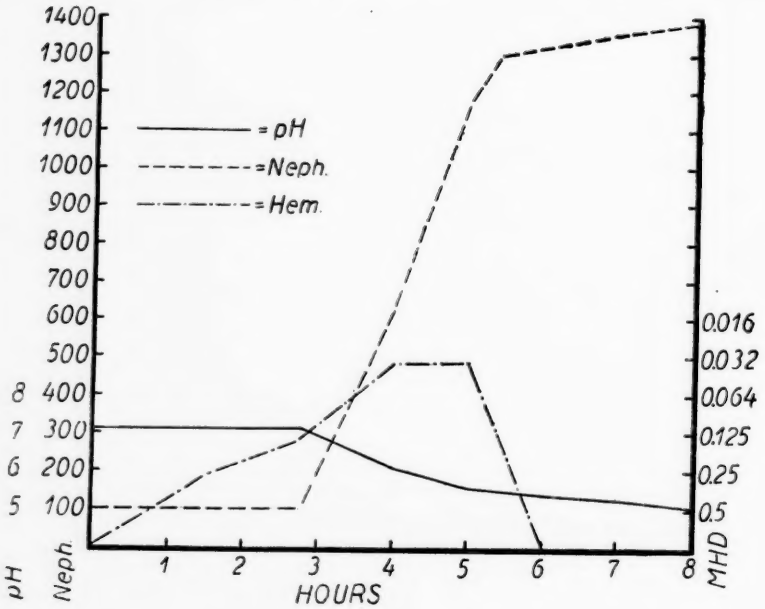

Fig. 3a. — Diagram showing hemolytic activity, pH and nephelometric measurement of bacterial growth during the production of colilysin in casein hydrolysate with yeast autolysate added.

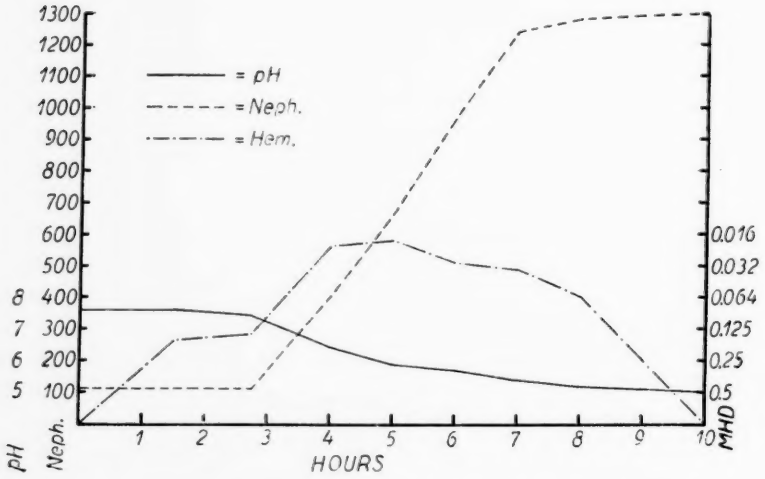

Fig. 3b. — Diagram showing hemolytic activity, pH and nephelometric measurement of bacterial growth during the production of colilysin in casein hydrolysate without yeast autolysate.

cent sterile solution each flask was inoculated with 2 ml. of a 16 hour sheep red cell-peptone water culture of strain No. 24 and placed in the thermostat at +37° C. Samples were taken with a sterile pipette immediately after inoculation and then at intervals of 2 hours. The samples were tested as in the foregoing experiment for determination of the hemolysis and pH. Nephelometric measurements of the turbidity of the medium were also made to evaluate the bacterial growth. The same tubes were used in all the nephelometric examinations and the mean of two readings was taken as the final value.

As will be seen from the diagrams in Fig. 3 a and b, the maximal hemolytic activity in both media occurred at 4 hours, although the turbidity had not then yet reached maximum. This occurred at about 7 hours. The nephelometric readings appeared to indicate that the production of hemolysin does not run parallel with the growth of bacteria but stops at the 4 hour maximum regardless of a marked increase in the turbidity of the medium. In the medium enriched with yeast autolysate, in particular, there was a rapid inactivation of the hemolysin after 5 hours' incubation regardless of increasing turbidity of the culture. In the other medium the inactivation of the hemolysin was somewhat slower. The changes in the pH values of the two media were concordant. At the time of commencement of diminished activity the pH value in both cases had dropped to below 6.0.

5. PRODUCTION OF COLI HEMOLYSIN IN ROBINSON'S MEDIUM

To obtain a clearer picture of the conditions required for the production of coli hemolysin, the culture medium used by Robinson (24) was compared with peptone water and casein hydrolysate, the media being compounded in 8 different ways. Eight 100 ml. Erlenmeyer flasks were filled with the following media:

No. 1: 1 per cent Witte's peptone water,
» 2: » » » » » + 0.5 per cent glucose,
» 3: » » » » » + 0.5 » » »
+ 0.02 per cent $CaCl_2$,
» 4: Casein hydrolysate + 0.5 per cent glucose,

No. 5: Robinson's defined medium without $CaCl_2$,
» 6: » » » with 0.02 per cent $CaCl_2$,
» 7: » » » + 1 per cent Witte's peptone,
» 8: » » » + 1 per cent Witte's peptone + 0.02 per cent $CaCl_2$.

The pH was adjusted to c. 7.5 and the flasks were autoclaved. The medium was then heated to c. + 30° C. and inoculated with 1.5 ml. of a 16 hour culture of strain No. 24 grown in sheep red cell-peptone water. Before the flasks were placed in the thermostat at + 37° C., 5 ml. was withdrawn in a sterile manner from each flask for determination of the pH, turbidity and hemolytic activity, as in the foregoing experiments. Samples were taken and tested similarly at intervals of 2 to 4 hours.

As is seen from Fig. 4 a, b and c, there was a marked difference between the production of hemolysin in the media containing

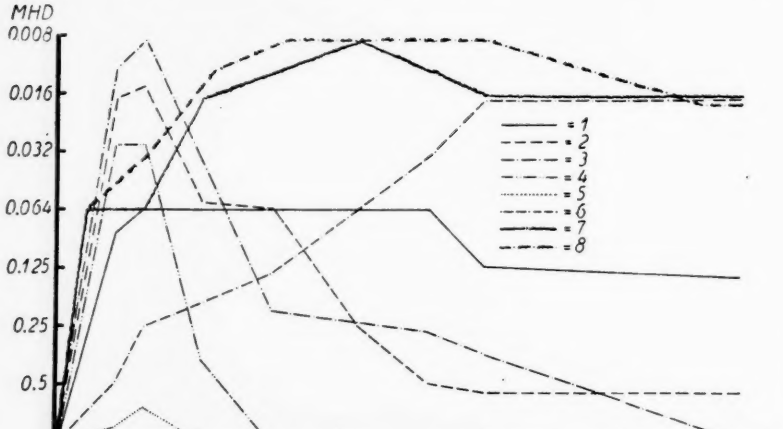

Fig. 4 a. — Diagram showing hemolytic activity of colilysin produced in various substrates. The substrates used were:

1 = 1 % peptone water
2 = » » » + 0.5 % glucose
3 = » » » + 0.5 % » + 0.02 % $CaCl_2$
4 = » casein hydrolysate + 0.5 % glucose
5 = » Robinson's medium without $CaCl_2$
6 = » » » with 0.02 % $CaCl_2$
7 = » » » + 1 % peptone
8 = » » » + 1 % » + 0.02 % $CaCl_2$

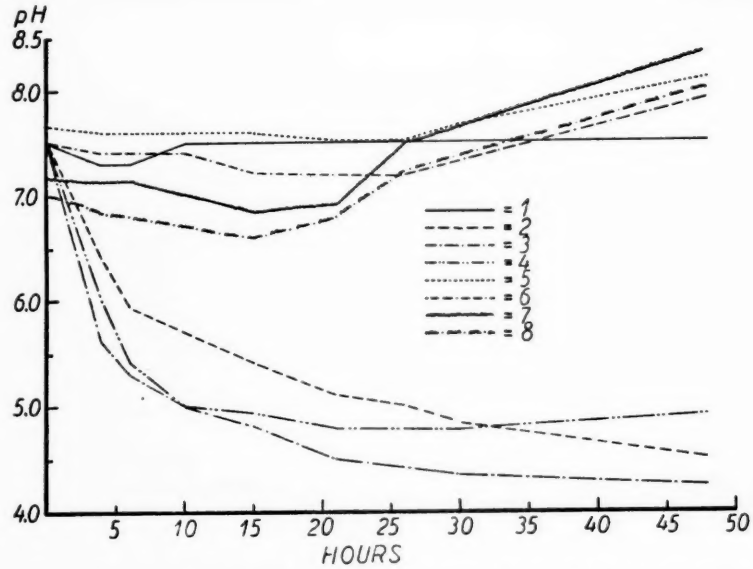

Fig. 4 b. — Diagram showing pH values of various media during the production of colilysin. Substrates as in Fig. 4 a.

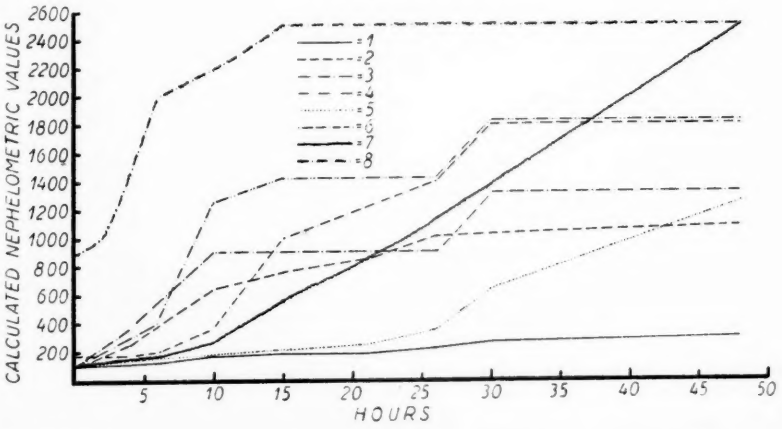

Fig. 4 c. — Diagram showing nephelometric measurement of turbidity of cultures in various media during the production of colilysin. Substrates as in Fig. 4 a.

glucose and in the different combinations of Robinson's defined medium. The addition of glucose accelerated the production of hemolysin, but also rendered the lysin considerably more labile. Calcium chloride added to the peptone water medium did not

enhance the hemolytic power to any degree worth mentioning, and the hemolysis probably should be ascribed to the original calcium content of the peptone. On the other hand, it was clearly evident that the addition of calcium ions was necessary for hemolytic activity in Robinson's medium. This bears out the findings in Robinson's own experiments.

The production of hemolysin in the different compounds of Robinson's medium was slower than in the other media used. The optimal hemolysis was not reached before c. 16 hours, as against 5 to 6 hours in the other media. The high degree of stability of the lysin in Robinson's medium, in association with constant and sometimes even rising pH values, is to be noted.

6. OTHER FACTORS INFLUENCING THE PRODUCTION OF COLI HEMOLYSIN

It has not been possible to confirm the assumption that a factor which inactivates the hemolysin is produced during bacterial growth. From lysin produced in peptone water from *Esch. coli* strain No. 24 the bacterial cells were centrifuged off in an International centrifuge at the rate of 15,000 r.p.m. for 15 minutes. The hemolytic activity of the lysin was C, C, C, C, (P) = 1: 16. Equal parts of this colilysin and of another colilysin, otherwise similar but 6 days old and fully inactive, were mixed. Before mixing, the inactive lysin was divided into two portions, from one of which the bacteria were centrifuged off, the other being used without centrifugation. For control, a similar amount of physiological saline was added to the active centrifuged lysin. The lysin mixtures tested were thus compounded as follows:

No. 1: 5 ml. centrifuged active lysin + 5 ml. non-centrifuged inactive lysin,
» 2: 5 » » » » +5 » centrifuged inactive lysin,
» 3: 5 » » » » +5 » physiological saline.

After the usual hemolysis controls had been made, test tubes containing the three mixtures were placed in the thermostat at + 37° C. The hemolytic activity was examined after 3 hours' and 18 hours' incubation and is shown in Table 4.

TABLE 4

HEMOLYTIC ACTIVITY OF MIXTURES OF ACTIVE AND INACTIVE COLILYSIN, AFTER INCUBATION AT +37° C. FOR 3 AND 18 HOURS

Mixture	Before Incubation	After 3 Hours	After 18 Hours
1	C, C, C, C, C, O = 1: 32	C, C, C, O = 1: 8	O, O
2	C, C, C, C, (P), O = 1: 16	C, C, C, O = 1: 8	(C), O = 1: 2
3	C, C, C, O = 1: 8	(C), O = 1: 2	O, O

The interesting observation can be made from the results that the mixtures of equal proportions of active and inactive lysin yielded before incubation a somewhat better hemolysis than that obtained with the active lysin alone, whereas the dilution of active lysin in physiological saline was only one-half as active as the original active lysin. After incubation in the thermostat at + 37° C. for 3 hours the hemolytic activity of all the lysin mixtures was greatly reduced, and after 18 hours they were completely inactive. Similar results were obtained with inactive lysins less than 6 days old.

Since no directly inactivating factor could be demonstrated in completely inactive lysins and they yielded, on the contrary, a slightly better hemolysis, additional tests were carried out with small amounts of centrifuged inactive lysin added to each of a series of hemolysis test tubes containing active lysin. Three series of tests for hemolysis were made with a peptone water-lysin which gave the hemolysis values of C, C, C, C, (P) = 1: 16. Varying amounts of inactive lysin were measured by pipette into the tubes in the different series before the addition of the red cell suspension.

TABLE 5

HEMOLYTIC ACTIVITY OF ACTIVE COLILYSIN AFTER ADDITION OF DIFFERENT AMOUNTS OF CENTRIFUGED INACTIVE LYSIN

Amt. of Inactive Lysin Added per Tube	Hemolytic Activity
—	C, C, C, C, (P), O = 1: 16
0.1 ml.	C, C, C, C, C, O = 1: 32
0.2 »	C, C, C, C, C, O = 1: 32
0.3 »	C, C, C, C, C, (P) = 1: 32

From the maximal hemolytic values listed in Table 5 it will be seen that, as in the preceding test, a slight increase in hemolytic activity was obtained, but that this increase in the activity was not enhanced by the use of larger amounts of inactive lysin.

7. EFFECT OF TEMPERATURE ON COLI HEMOLYSIN

Centrifuged peptone water-colilysin obtained from strain No. 24 was distributed into 20 WaR tubes in amounts of 1 ml. The tubes

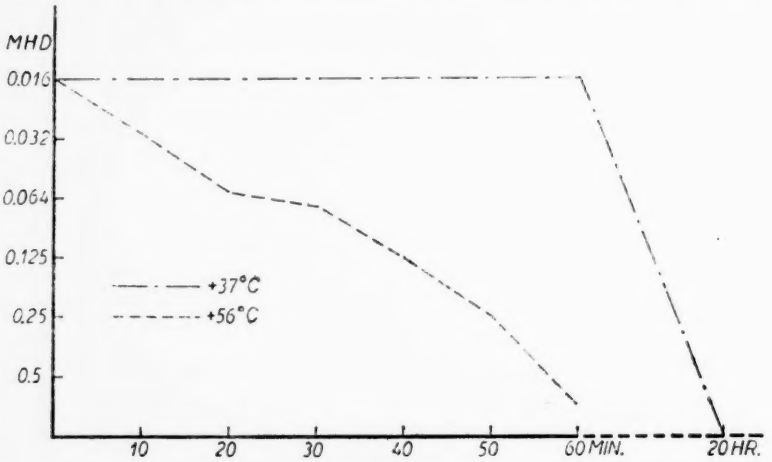

Fig. 5. — Diagram showing hemolytic activity of centrifuged peptone water-colilysin at +37° C. and +56° C.

were placed in 2 test tube racks, which were then immersed in the water bath at + 37° C. and + 56° C., respectively. Before immersion 1 tube was removed from each rack for evaluation of the hemolytic activity. This was repeated at intervals of 10 minutes.

The graph in Fig. 5 shows that at + 56° C. the lysin was inactivated within 1 hour, whereas a temperature of + 37° C. had no effect on the activity within this time but did inactivate the lysin in 20 hours. The conclusion may therefore be drawn that the coli hemolysin is thermolabile, which confirms the finding by Robinson (24).

8. SEPARATION OF COLI HEMOLYSIN FROM THE BACTERIAL CELLS

All earlier attempts to separate the lysin from bacteria by means of various bacterial filters have been unsuccesful. Both

Sjöstedt (29) and Robinson (24) endeavored to employ various methods of separation at different degrees of pH, also by adding reducing agents, such as sodium dithionite used in the preparation of streptolysin.

Since the optimal conditions for hemolytic activity in the different substrates and the lability of the lysin are known, the importance of removing the bacteria from the substrate at the right time and as rapidly as possible is self-evident. For separation I have used the following procedure. The bacterial culture was taken from the + 37° C. thermostat at the optimum of hemolysin production and was immediately centrifuged in an International centrifuge at ± 0° C. for 30—40 minutes at c. 3,000 r.p.m. Sterilized 250 ml. centrifuge bottles were used for this purpose.

This treatment yielded a completely clear lysin with its activity retained. My efforts to pass the lysin-bearing liquid through a Seitz-Chamberland or porcelain filter resulted in complete inactivation of the lysin.

When the bacterial mass remaining in the centrifuge bottles was precipitated with sterile physiological saline to one-tenth of the original volume and re-centrifuged, a very slight hemolysis was obtained in 1 tube. An attempt was made to break the bacterial cells and to release a possible endotoxin by the method employed by Oker-Blom (21) in his work with pneumolysin. A bacterial suspension consisting of the bacteria from 200 ml. centrifuged lysin in 20 ml. physiological saline was alternately frozen in a mixture of dry ice and alcohol and thawed at + 37° C. ten times in succession. A hemolysis test made after centrifugation of the liquid showed hemolysis in 1 tube only.

Attempts to release colilysin from the bacterial cells by subjecting the bacterial suspensions to ultrasonic waves gave completely negative results.

The results of these experiments seem to indicate that the coli hemolysin probably is not bound to the bacterial cells but is evidently of the nature of an exotoxin.

The substrate chiefly used in this work is a compound of 1 per cent Witte's peptone water and 0.5 per cent glucose, pH 7.6. The medium compounded by Robinson (24), it is true, yielded a more stable lysin, but since a considerable part of my tests had already been carried out before the publication of Robinson's report it was

no longer possible to change the medium. Furthermore, repeated attempts to centrifuge a 24 hour old »Robinson's lysin» in the same manner as the lysin produced in peptone water resulted in complete inactivation of the lysin. For example, a 24 hour old »Robinson's lysin» with a hemolytic activity of C, C, C, C, C, P = 1: 64 was divided into two portions. One of the portions was centrifuged in a Spinco centrifuge in vacuum for 15 minutes at 15,000 r.p.m. The hemolysis test made with the supernatant fluid after centrifugation gave the result of (C), O. Assuming that the vacuum may possibly have a reducing effect, the other portion was centrifuged in an International centrifuge for 20 minutes at 3,000 r.p.m. at a temperature of + 1° C. The result was the same as for the first portion. Attempts to suspend the bacterial mass in one-tenth volume of physiological saline and to centrifuge the fluid once more did not yield hemolysis. From these failures it was concluded that centrifugation inactivated the lysin produced in Robinson's medium, since no hemolytic activity could be demonstrated in the supernatant fluid centrifuged from the bacterial mass suspension. For this reason it was not possible carry out comparative titrations of patient sera with lysin prepared in Robinson's medium.

Since it therefore was not possible to separate the lysin from the bacteria by methods other than centrifugation, I have in the present investigation mostly used lysin prepared in this way. The lysins used in the later stages of the work for the immunization of rabbits and the titration of patient sera were centrifuged in a high speed centrifuge for 15 minutes at 15,000 r.p.m. in order to produce a lysin that would be as free from bacterial cells as possible.

9. PRECIPITATION AND CONCENTRATION OF COLI HEMOLYSIN

As efforts to produce a fully sterile coli hemolysin by the different methods generally in use at present have been unsuccessful, tests were made to precipitate the lysin by various methods for protein precipitation.

a. Precipitation with Methyl Alcohol at —10° C.

As the optimal conditions required for precipitation of colihemolysin were unknown, a batch of well centrifuged lysin produced in casein hydrolysate medium was distributed in amounts

of 50 ml. into five 200 ml. Erlenmeyer flasks. The initial pH of the lysin was 6.2 and it was adjusted with N./10 HCl to 4.0, 4.5, 5.0, 5.5 and 6.0, respectively. The usual hemolysis control samples were taken and the lysin in the flasks was cooled to ±0° C. in an alcohol bath at —10° C. Then methyl alcohol at —10° C. was added dropwise into each flask with constant stirring and control of the temperature, which was not permitted to rise over ±0° C. After the addition of 30 ml. of methyl alcohol a distinct turbidity was seen in the flasks. The addition of alcohol was continued until each flask contained 60 ml. of CH_3OH and 50 ml. of lysin. One-half hour after the last drops of alcohol were added the flasks were removed from the alcohol bath and the contents were centrifuged in cooled centrifuge bottles for 20 minutes at a temperature of — 10° C. and at the rate of 3,000 r.p.m.

The supernatant fluid was discarded and the residual grayish sediment was suspended in 10 ml. of physiological saline. After storage for 1 hour in the refrigerator at + 4° C. the different portions were centrifuged once more and clear solutions were obtained.

TABLE 6 a

HEMOLYTIC ACTIVITY OF 50 ML. COLILYSIN AT DIFFERENT PH LEVELS

pH	Hemolysin Dilution							
	1: 2	1: 4	1: 8	1: 16	1: 32	1: 64	1: 128	1: 256
4.0	C	C	C	C	(P)	O	O	O
4.5	C	C	C	C	C	P	O	O
5.0	C	C	C	C	C	P	O	O
5.5	C	C	C	C	C	P	O	O
6.0	C	C	C	C	C	P	O	O

TABLE 6 b

HEMOLYTIC ACTIVITY AT DIFFERENT PH LEVELS OF PRECIPITATES FROM 50 ML. COLILYSIN PRECIPITATED WITH 60 ML. METHYL ALCOHOL AT —10° C.

pH	Hemolysin Dilution							
	1: 2	1: 4	1: 8	1: 16	1: 32	1: 64	1: 128	1: 256
4.0	C	C	C	C	C	P	O	O
4.5	C	C	C	C	C	C	C	P
5.0	C	C	C	C	C	C	C	O
5.5	C	C	C	C	C	C	C	O
6.0	C	C	C	C	C	C	C	O

A comparison of the initial hemolysis tests and the results of the tests with the 5 precipitates are shown in Tables 6 a and b. These results indicate that coli hemolysin can be precipitated and concentrated by means of methyl alcohol at a low temperature, and that the optimum pH level for this method lies between pH 4.5 and 5.0, as was also the case in a number of other experiments.

For the purpose of determining the approximate optimal amount of methyl alcohol to be used at pH 4.5, a similar series of precipitations was carried out with 5 portions of 50 ml. casein hydrolysate-lysin. After adjustment of the pH to 4.5 before precipitation, the hemolytic activity of the lysin was C, C, C, C, C, O = 1: 32. When methyl alcohol was added to the different portions in amounts of 50, 100, 150, 200 and 250 ml. and precipitations were carried out as above, the results shown in Table 7 were obtained. It will be seen that for maximal effect the volume of methyl alcohol may not greatly exceed the amount of lysin under the described conditions of precipitation.

The precipitations were repeated under the same conditions and using the same amounts of lysin but with smaller variations in the amounts of methyl alcohol used.

The initial hemolysis produced by this lysin after adjustment of pH to 4.5 was: C, C, C, C, C, (P) = 1: 32. Table 8 shows the hemolysis obtained with the precipitates.

These results indicate that lysin is precipitated already after the addition of one-fifth volume of methyl alcohol and that the yield is increased in all mixtures up to the 1: 1 ratio of methyl alcohol and lysin. Any excess of methyl alcohol over this amount

TABLE 7

HEMOLYTIC ACTIVITY OF PRECIPITATES FROM 50 ML. COLILYSIN PRECIPITATED WITH DIFFERENT AMOUNTS OF METHYL ALCOHOL AT —10° C. AT PH 4.5.

Amt. of CH_3OH	Hemolysin Dilution							
	1: 2	1: 4	1: 8	1: 16	1: 32	1: 64	1: 128	1: 256
50 ml.	C	C	C	C	C	C	P	O
100 ml.	C	C	C	C	C	C	O	O
150 ml.	C	C	C	C	C	O	O	O
200 ml.	C	C	C	C	C	O	O	O
250 ml.	C	C	C	C	P	O	O	O

TABLE 8

HEMOLYTIC ACTIVITY OF PRECIPITATES FROM 50 ML. COLILYSIN PRECIPITATED WITH SLIGHTLY DIFFERENT AMOUNTS OF METHYL ALCOHOL AT —10° C. AT PH 4.5.

Amt. of CH_3OH	Hemolysin Dilution						
	1: 2	1: 4	1: 8	1: 16	1: 32	1: 64	1: 128
10.0	C	C	C	C	(P)	O	O
20.0	C	C	C	C	(P)	O	O
30.0	C	C	C	C	P	O	O
40.0	C	C	C	C	P	(P)	O
50.0	C	C	C	C	C	C	O
60.0	C	C	C	C	C	C	O
70.0	C	C	C	C	C	C	O
80.0	C	C	C	C	C	C	O

does not increase the amount of lysin precipitated. Corresponding control precipitations of the culture medium alone failed to demonstrate any hemolytic activity.

A volume of solvent equal to at least one-tenth of the original volume of the lysin was found suitable for solution of the lysin precipitate. Both water and physiological saline was tested for this purpose and the latter was found to allow a better hemolytic activity and more thorough solution of the precipitate. After renewed solution of the residual precipitate in physiological saline and re-centrifugation, a good hemolytic activity was still demonstrable. If solution and centrifugation were repeated for a third time, the supernatant fluid still showed hemolytic activity.

This is clearly shown by an experiment in which 700 ml. of lysin prepared in casein hydrolysate and giving an initial hemolysis of C, C, C, C, C, (P) = 1: 32 was precipitated with 700 ml. of CH_3OH at pH 4.5 at — 10° C. After centrifugation the precipitate obtained was dissolved in 35 ml. of physiological saline. Supernatant fluid No. 1 yielded hemolysis as follows: C, C, C, C, C, C, C, P = 1: 256. The precipitate was once more dissolved in 20 ml. of physiological saline and centrifuged. The resulting supernatant fluid No. 2 gave a hemolysis of C, C, C, C, C, C, P = 1: 128. A third solution made in the same amount of physiological saline yielded after centrifugation the hemolysis values of C, C, C, C, C, P = 1: 64.

The examples given above illustrate clearly how difficult it is to liberate the precipitated lysin from the protein precipitate obtained. No improvement was seen in the results when N./10 Sörensen's phosphate buffer solution at variable pH values of 5, 6 and 7 was substituted for physiological saline and H_2O. No other buffers were tested. Similar results were obtained by the precipitation of coli hemolysin prepared from a peptone water culture, but this medium was not used for the reason that the peptone accompanies the precipitate.

Regardless of the precipitation and concentration of the lysins in this manner it was not possible to stabilize them, and they retained their labile nature to nearly the same degree as previously.

An attempt was made to obtain by electrophoresis some idea of the composition of the colilysin, but regardless of precipitation twice with alcohol the lysin could not be obtained in a sufficiently purified form to permit any conclusion from the electrophoretic curve.

b. Precipitation with Ammonium Sulphate

Lysin prepared from strain No. 24 grown in 1 per cent peptone water with glucose and giving an initial hemolysis of C, C, C, C, C, C, O = 1: 64 was distributed into 4 Erlenmeyer flasks, each containing 50 ml. The pH values were adjusted with N./10 HCl solution to 4.0, 4.5, 5.0 and 5.5. The flasks were placed in the refrigerator at —3° C. When the temperature of the lysin was $\pm$ 0° C., 7 volumes of a saturated solution of $(NH_4)_2\ SO_4$ were added dropwise to 3 volumes of lysin, stirring constantly. This produced a powerful precipitation. After storage for 1 hour in the refrigerator, the contents of the flasks were centrifuged for 20 minutes at 3,000 r.p.m. at a temperature of — 2° C. The supernatant particles and the precipitate were dissolved together in 10 ml. of H_2O. After vigorous stirring of the precipitate with a glass rod, the centrifugation was repeated. The hemolysis then obtained with the different precipitates is shown in Table 9.

This test has shown that a 70 per cent saturated solution of $(NH_4)_2\ SO_4$ can also be employed for the precipitation of the coli-

TABLE 9

HEMOLYTIC ACTIVITY OF COLILYSIN PRECIPITATE OBTAINED BY PRECIPITATION WITH $(NH_4)_2\ SO_4$ AT $\pm$ 0° C. AT DIFFERENT PH LEVELS.

pH	Hemolysin Dilution							
	1: 2	1: 4	1: 8	1: 16	1: 32	1: 64	1: 128	1: 256
4.0	C	C	C	C	C	C	(P)	O
4.5	C	C	C	C	C	C	C	(P)
5.0	C	C	C	C	C	C	C	O
5.5	C	C	C	C	C	C	C	O

lysin from the peptone water-lysin. The optimal pH of this test, also, was 4.5. Control precipitations made of the peptone water alone under identical test conditions gave no hemolysis.

Because of the lability of the lysin, dialysis of the peptone water-lysin did not prove possible without loss of the hemolytic activity. The addition to the dialyzed fluid of calcium ions in various concentrations of a $CaCl_2$ solution, or of a phosphate buffer solution had no effect on the reduced activity. For this reason no attempts were made to precipitate with $(NH_4)_2\ SO_4$ the colilysin produced in other media.

10. LYOPHILIZATION OF COLI HEMOLYSIN PRECIPITATE

As the precipitated and re-dissolved coli hemolysin proved to be as labile as it had been before precipitation, experiments were carried out for the drying of the lysin in vacuum in a lyophilizer.

The precipitate from casein hydrolysate-lysin was placed in a 250 ml. globular flask, which was immersed in a mixture of dry ice and alcohol at —73° C. The flask was constantly rotated so that a thin layer was formed by the freezing liquid on the inside wall of the flask. The flask was then connected for c. 14 hours to a refrigerated condenser and vacuum pump, at a pressure of 0.05 mm. Hg. A fine dust-like white powder was obtained, which was readily soluble in water and had a high hemolytic power. Storage of the powder for 8 months in a vacuum-closed ampoule at a temperature of —18° C. maintained the hemolytic activity unchanged.

The maximal amount of powdered active colilysin obtained in this experiment by the precipitation and freeze-drying processes

was c. 30 per cent of the original quantity of active lysin, determined by means of hemolysis tests. The concentration of the lysin had increased to 2- or 3-fold.

In view of the complicated and time-consuming process required and of the relatively small amounts that can be handled at one time, purified lysin was not used in the clinical tests made in this investigation.

11. STORAGE OF COLI HEMOLYSIN

The storage of coli hemolysin proved one of the greatest difficulties throughout the present work. The lability of the lysin and its instability at room temperature became evident already in the first stages of the experiments. Streptolysin and staphylolysin remain unchanged for nearly 3 months if stored in sterile bottles at + 4° C., but the stability of the colilysin was not improved to any appreciable degree by storage at this temperature. Centrifuged peptone water-colilysin was therefore placed into sterile rubber-stoppered test tubes immediately after centrifugation and kept at a temperature of —18° C. When thus stored, the lysin retained its hemolytic power practically unchanged for 10 days, after which a marked reduction in the activity set in.

The addition of various substances with a possible stabilizing effect was therefore tested. For this purpose 25 per cent sterile glycerin solution, 0.1 per cent cysteine and 0.1 per cent sodium thioglycolate were each added to a different portion of peptone water-lysin in globular flasks. Lysin with no addition was used as control. The four flasks were placed at a temperature of — 18° C. and hemolysis controls were made after 10, 17 and 20 days.

As will be noted from Fig. 6, the addition of 25 per cent of sterile glycerin solution gave the lysin increased stability, whereas the portions to which the reducing agents cysteine and sodium thioglycolate had been added showed a decline in hemolytic activity which was, on the whole, parallel with that in plain lysin. Other reducing agents, such as sodium hydrosulphite and ascorbic acid, were also tested, but with equally negative results.

The effect of the pH level on the stability of the lysin was studied by adjusting the pH of a centrifuged peptone water-lysin to 4.0, 5.0, 6.0, 7.0 and 8.0, respectively, by means of N./10 Sören-

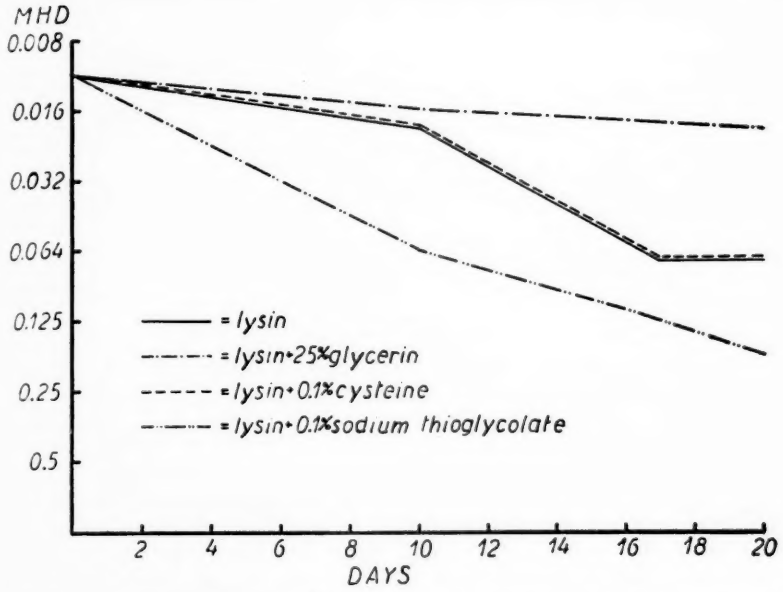

Fig. 6. — Diagram showing storage at —18° C. of centrifuged peptone water-colilysin, with and without various added substances.

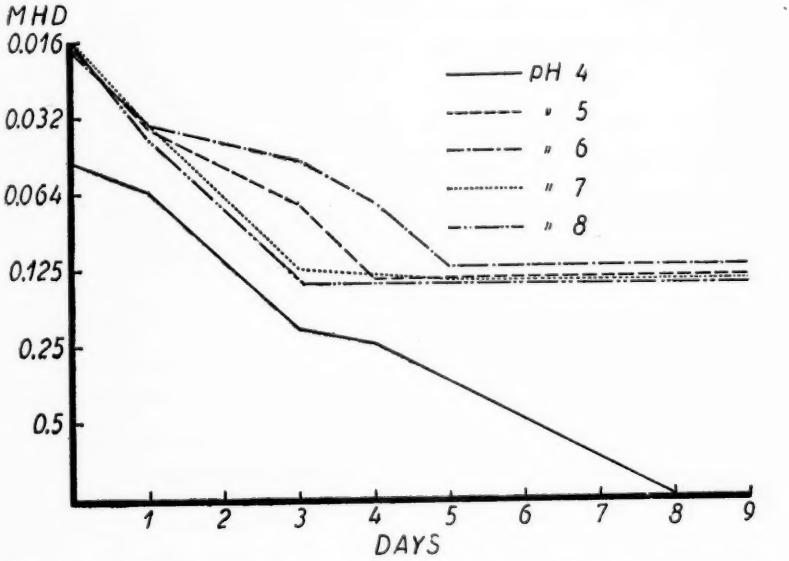

Fig. 7. — Diagram showing storage at +4° C. of centrifuged peptone water-colilysin at various pH levels.

sen's phosphate buffer. After determination of the initial hemolytic activity the 5 portions were placed in sterile tubes in the refrigerator at + 4° C. Tests for hemolytic activity were carried out at equal intervals and the results are shown in Fig. 7.

As shown, the lysin at pH 4.0 became completely inactive in 8 days, whereas the activity of the other 4 portions showed an 8-fold reduction after 5 days' storage at + 4° C. The reduction in activity then proceeded at a slower rate and total inactivity was reached in 12—14 days.

The best results in the storage of coli hemolysin were obtained with lysin produced in casein hydrolysate, precipitated with alcohol, freeze-dried, and stored in vacuum-sealed ampoules at —18° C. (cf. page 35). It was possible to store lysin of this kind for 8 months with no change in the hemolytic activity, but in view of the complicated method involved and the small yield from the precipitation this lysin was not employed for more extensive tests.

C. SUMMARY

In preliminary tests, coli hemolysin was prepared from hemolytic strains of *Esch. coli* in ordinary beef broth and in different modifications of 1 per cent Witte's peptone water, of casein hydrolysate, and of a medium defined by Robinson (24). For the continued experiments the colilysin was produced in 1 per cent Witte's peptone water with 0.5 per cent glucose added, and in casein hydrolysate solution with 0.5 per cent glucose.

The potency of coli hemolysin was tested serologically with a 2 per cent suspension of three times washed sheep red cells and was expressed as MHD (minimum hemolytic dose).

In view of the high lability of colilysin, experiments were made for the production of lysin in O_2 and N_2 atmospheres. However, the gases were not seen to exert any direct influence on the production and stability of the lysin. On the other hand, there seemed to exist a certain degree of correlation between the declining pH of the culture and the diminishing activity of the colilysin.

The lysin production did not seem to increase parallel to the nephelometrically evaluated growth of bacteria in the medium.

It was not possible to separate the coli hemolysin from the bacterial culture by filtration through various bacterial filters at different pH levels or in different O_2 atmospheres. Breaking up of the centrifuged bacterial mass by repeated freezing and thawing and by ultrasonic waves also failed to yield active lysin. It therefore appears probable that coli hemolysin is of the nature of an exotoxin.

A clear, hemolytically active colilysin was obtained by high-speed centrifugation from lysins produced in peptone water and in casein hydrolysate solution. Centrifugation appeared to inactivate the lysin produced in Robinson's medium.

Active colilysin was also obtained by precipitation of peptone water-lysin with a saturated solution of $(NH_4)_2\ SO_4$ at $\pm 0°$ C. However, attempts to dialyze the resulting precipitate against H_2O and yet retain an unchanged hemolytic activity were unsuccessful.

Active colilysin was precipitated with methyl alcohol from centrifuged casein hydrolysate-lysin, pH 4.5, at —10° C. Solution of the lysin precipitate was difficult in H_2O, physiological saline solution, and N./10 Sörensen's phosphate buffer at varying pH levels.

Freeze-drying (lyophilization) of colilysin separated by precipitation with methyl alcohol increased the concentration of the lysin 2- to 3-fold. The yield of active lysin in powdered form was c. 30 per cent of the original amount of active lysin.

No hemolytic activity was obtained in parallel precipitations of the substrates alone.

It has been found that colilysin is a thermolabile lysin. Total inactivation occurred in 1 hour at + 56° C. and in 20 hours at + 37° C.

Storage of colilysin at +4° C. was not possible for over 24 hours without decrease in the hemolytic activity. However, the activity was maintained unchanged for 10 days at —18° C. The addition of a 25 per cent sterile solution of glycerin stabilized the hemolytic activity and increased the storage time at — 18° C. to 21 days. Alcohol-precipitated and freeze-dried lysin stored in powdered form in vacuum-sealed ampoules at — 18° C. retained its hemolytic power for 8 months.

CHAPTER III

SEROLOGICAL STUDIES ON ANTICOLILYSINS

A. COLILYSIN AND ANTICOLILYSIN IN ANIMAL EXPERIMENTS

1. IMMUNIZATION OF RABBITS

For the study of specific anticolilysins I carried out immunization experiments with 2 strains of *Esch. coli* using 2 rabbits for each strain. Before immunization, a serum sample was taken for the determination of possible initial inhibition of hemolysis. The lysins used in the experiments were produced in peptone water medium from 2 strains belonging to O groups 2 and 54, respectively. A new batch of lysin was prepared and centrifuged on each day of immunization.

The hemolytic power of the lysins used for the immunization injections was as follows, expressed as MHD:

Lysin from strain No. 5408, O group 2 .. 0.032—0.016 MHD
» » » » 5598, » » 54 .. 0.064—0.032 »

For both lysins the first immunizing dose was 0.3 ml. It was then raised to 0.5—0.75—1.0—1.5—2.0—2.5 . . . . . 5.0 ml. Injections were made intravenously into the animal's ear 3 times a week, with no immediate untoward reactions whatsoever. When the dosage of 5.0 ml. was reached, it was not increased and further injections were made at this level.

About 5 ml. of blood was withdrawn every 10th day from the animal's ear by venipuncture. The sample was kept at + 4° C. overnight and was centrifuged on the following day. The serum was inactivated at +56° C. for $^1/_2$ hour and stored in sterile tubes at — 18° C.

The immunization period was 50 days. A longer period could not be used, as the ear veins became thrombosed. On the 50th day c. 50 ml. blood was withdrawn by heart puncture for use as a standard serum.

Routine blood samples were taken up to the 80th day from commencement of immunizations. Rabbit No. 419, immunized with lysin from O group 2, died on the 67th day in a greatly emaciated condition. The other rabbits, on the whole, kept up their initial weight.

2. TITRATION OF ANTICOLILYSIN IN RABBIT SERA

The titration of anticolilysin in the sera of the rabbits was carried out along the principles followed by Oker-Blom (21) in his investigation on pneumolysin.

Inactivated serum was diluted 1: 50, 1: 100, 1: 200 . . . . . 1: 1600 in 1.0 ml. of physiological saline solution. The MHD of the lysin to be used was determined prior to each titration by means of the usual hemolysis test series. The lysin was then diluted with physiological saline solution to a potency of 10 × MHD, and 0.5 ml. of this dilution was added to each tube. Titrations with smaller amounts of colilysin were also attempted but the dose of 10 × MHD gave the most distinct readings. The tubes with the serum-lysin mixture were shaken and placed in a water bath at + 37° C. for 15 minutes for fixation. To each tube was then added 0.5 ml. of a 2 per cent suspension of three times washed sheep red cells in physiological saline solution. After thorough shaking, the tubes were incubated in the water bath at + 37° C. for 45 minutes. When some sedimentation of the blood cells had taken place at room temperature, readings were taken 1 hour after removal of the tubes from the water bath by noting the tube preceding the first tube with partial (over c. 50 per cent) or complete hemolysis.

The following is an example of a reading:

Serum dilution	1: 50	1: 100	1: 200	1: 400	1: 800	1: 1600
Degree of hemolysis ..	O	O	O	O	P	C

O = no hemolysis
P = partial hemolysis
C = complete hemolysis

The anticolilysin titer of the serum in this case was 1: 400.

The course of the immunization was followed by anticolilysin titration of the sera of the immunized rabbits with homologous lysin, with the results shown in Fig. 8. The maximal immunization was reached on the 50th day, when injections had to be discontinued, as stated above. The best immune serum was obtained with O 2 lysin, which gave an antihemolytic titer of 1: 1200 with homologous lysin. The highest titer for immune serum from lysin

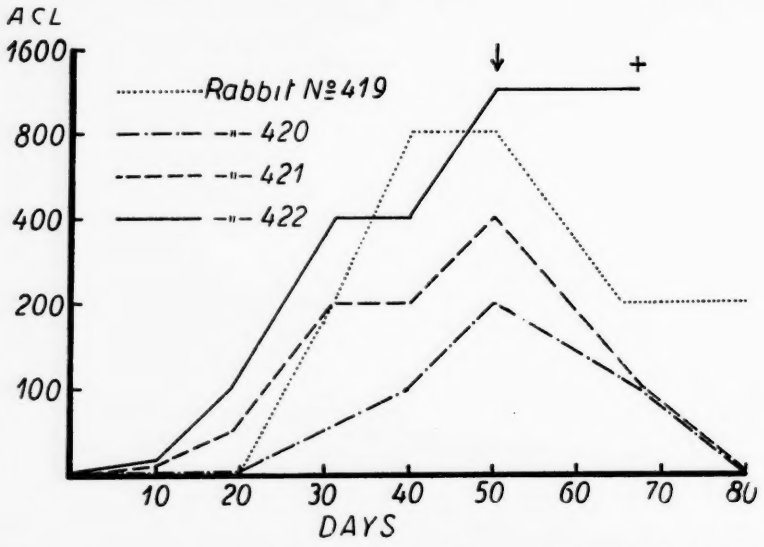

Fig. 8. — Diagram showing production of anticolilysin in the sera of rabbits during the immunization experiment with colilysin from 2 strains of different O groups. The colilysins used were: Rabbits No. 419 and 420 — a strain of O group 2; rabbits No. 421 and 422 — a strain of O group 54. Arrow indicates time of discontinuance of immunization injections and taking of samples for standard serum. Cross indicates death of rabbit No. 419.

O 54 was 1: 800. It also appears from the graph that when immunization was discontinued, a marked drop took place in the antihemolytic power of the sera, and at 80 days sera No. 420 and 421 had no inhibitory effect on hemolysis. For serum No. 422 the titer still was 1: 200 at that time.

At the end of the experiment on the 80th day, all the stored sera from the immunized rabbits were titrated both with homologous lysin and with heterologous lysin from strain No. 24 causing agglutination in sera O 6, O 33 and O 87. The results of the titrations are seen in Table 10.

TABLE 10

ANTICOLILYSIN CONTENT OF RABBIT SERA TITRATED WITH HOMOLOGOUS AND HETEROLOGOUS COLILYSIN DURING IMMUNIZATION EXPERIMENT

Lysin	Rabbit No.	Days After Commencement of Immunization							
		0	10	20	32	40	50	67	80
Homologous	419	0	0	100	400	400	1200	1200	—
	420	0	0	50	200	200	400	100	0
	421	0	0	0	50	100	200	100	0
	422	0	0	0	200	800	800	200	200
Heterologous	419	0	0	0	400	800	1600	1600	—
	420	0	0	0	200	200	200	100	0
	421	0	0	0	100	100	400	100	0
	422	0	0	0	400	800	800	200	200

It will be noted that, on the whole, there is uniformity in the titers obtained with the two lysins. It therefore appears that coli hemolysin may be regarded as an antigen which, during repeated injections into rabbits, will give a rising anticoli titer that gradually declines when injections are discontinued.

The specificity of the anticolilysins in relation to other bacterial hemolysins was investigated by titration of the above mentioned standard immune rabbit sera with staphylolysin and streptolysin. The results are shown in Table 11.

TABLE 11

ANTISTREPTOLYSIN, ANTISTAPHYLOLYSIN AND ANTICOLILYSIN TITERS OF SERA OF RABBITS IMMUNIZED WITH COLILYSIN

Rabbit No.	Antistreptolysin	Antistaphylolysin	Anticolilysin
419	< 50	< 0.32	1600
420	< 50	< 0.32	200
421	< 50	< 0.32	400
422	< 50	< 0.32	800

It is seen from this table that the anticolilysin sera had no inhibitory effect when titrated with streptolysin and staphylolysin, whereas the same sera in titrations with heterologous colilysin had an inhibitory power in a concentration as low as 1: 1600.

B. ANTICOLILYSIN IN »NORMAL» HUMAN SERA

1. METHOD OF TITRATION

Since the hemolytic activity of colilysin is variable and of low stability, as was seen in Chapter II, and since efforts to stabilize the lysin were unsuccessful, it was not possible to use the MHD alone as measure of the hemolytic activity. For this reason, in all the examinations of human sera, parallel titrations were made with certain standard sera of known titer, as has been done, for instance, in AST and Asta reactions.

In view of the instability of the lysin, a new batch of coli hemolysin was prepared before each titration by the peptone water method described in Chapter II, using the same strain (No. 24) in all cases. After centrifugation and storage overnight at ± 0° C., the MHD determination was made. As was done in the titrations with rabbit serum described above, a colilysin dose of 10 × MHD was used in the clinical examinations. Since the hemolytic activity of the lysin could not be standardized for any great length of time, each titration included 3 different antisera of known titers, determined as the mean of 10 successive titrations. Standard sera No. 419 and 268 were rabbit sera obtained from the immunization experiments (cf. Chapter III A) and serum »Ar» was from a patient with a bacteriologically confirmed septicemia caused by *Esch. coli* (cf. page 58). The mean titers for these three sera were as follows: No. 419 = 1: 1200, No. 268 = 1: 800, and »Ar» = 1: 6400.

If in the tests on human sera the standard sera did not give the original titers, the titration procedure was repeated. The method of titration used in the clinical tests was the same as in the rabbit immunization tests.

The titrations were carried out with a routine series of 6 tubes in falling serum concentrations of 1: 50, 1: 100 . . . . . 1: 1600. If hemolysis was inhibited in all the tubes, the titrations were repeated with a longer series of dilutions. The readings were taken as described in connection with the tests with rabbit serum. To simplify the reporting of results, the anticoli titer is designated hereafter by the term ACL, in analogy to the established designations for AST and Asta reactions.

2. »NORMAL» SERIES

In the »normal» series a total of 206 sera from »healthy» persons who had had no infectious diseases during the preceding 3 months were examined. Most of the sera were received from the blood bank of the Finnish Red Cross; the remainder came from homes for children or were taken from medical students. Persons with a case history of various intra-abdominal or urinary tract infections were eliminated. The sera were divided into groups according to the age of the subjects, each 10-year group containing a minimum of 20 sera.

In order to give a more balanced presentation of the distribution of the ACL values of the normal sera, the graph in Fig. 9 is drawn up by plotting in the abscissa the tube numbers instead of the corresponding serum dilutions. Thus, for instance, tube No. 1 corresponds to a dilution and titer of 1: 50, No. 2 to 1: 100, a.s.o.

The children's series, consisting of the sera of 38 children aged 1—4 years, is seen to be distributed into two main groups at tube 1 and around tube 4, whereas the majority of the other sera are grouped at tubes 2 and 3. The curve representing the mean values for all the age groups runs fairly low up to the age of 45, after which it rises with advancing age. The increased titers seen with older persons are probably accounted for by a relatively higher incidence of latent colibacillary infections in elderly persons, as found, *e.g.*, by H. Renkonen (23) in diseases of the gallbladder (cf. Table 15).

TABLE 12

DISTRIBUTION OF ACL TITERS OF 206 »NORMAL» SERA

A C L										
50	75	100	150	200	300	400	600	800	>400 (%)	$\leqq 400$ (%)
30	2	39	14	73	6	25	9	8	8 %	20 %

For determination of the normal ACL limit, this series is classified by titers in Table 12. It will be seen that 92 per cent of the persons in the normal series had an ACL of 400 or less (ACL $\leqq 400$) and that only 8 per cent were over 400 (ACL > 400).

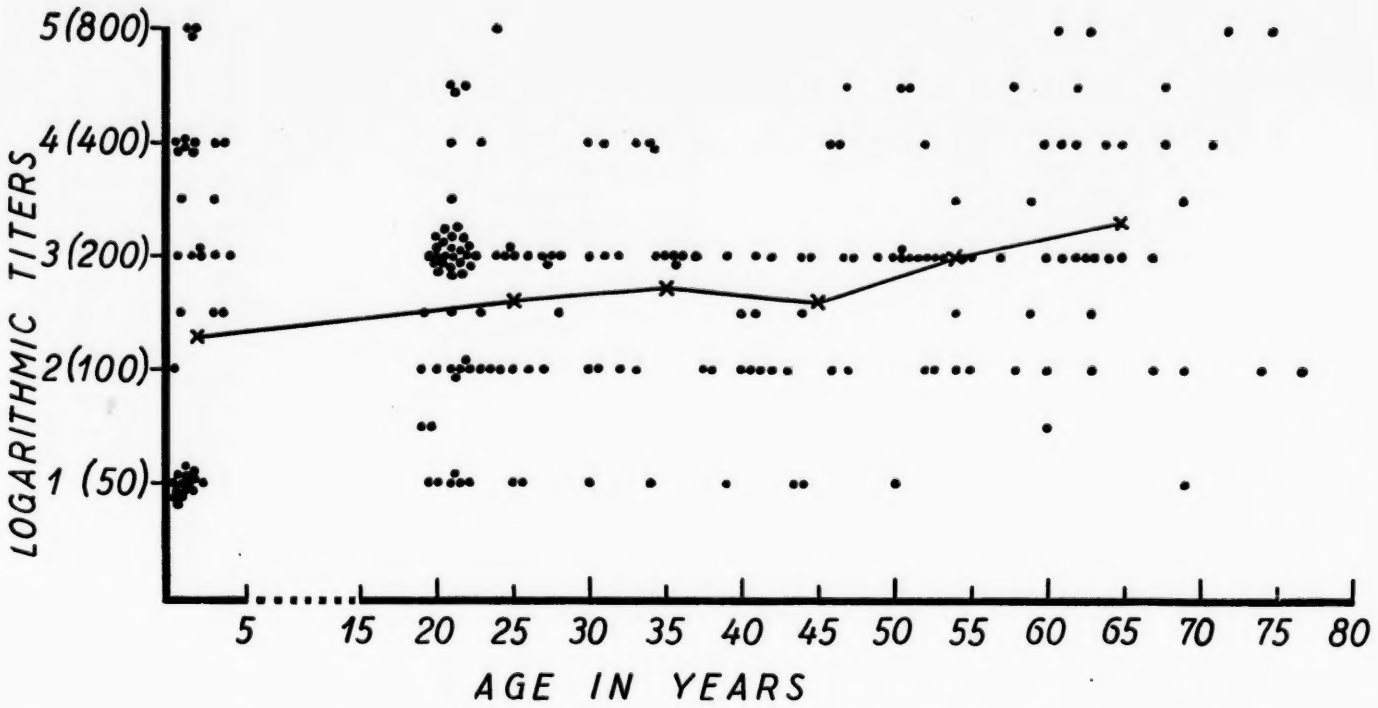

Fig. 9. — Diagram showing distribution of 206 »normal» sera by age of patients and anticolilysin titers (ACL).

The titers over 400 may have been due, for instance, to the presence of colibacillary infections overlooked in the case histories, changes in the lipid fraction of the serum (Neill, *et al.*, 20, Oker-Blom, *et al.*, 22), or to a higher incidence of latent infections with *Esch. coli* in older persons. However, in consideration of the percentages obtained in this »normal» series, ACL values over 400 are probably to be regarded as elevated above the normal and a value of 400 as moderately elevated. The incidence of ACL values of 400 and over (ACL $\geqq$ 400) in the »normal» series was 20 per cent.

Since the incidence of urinary tract infections caused by *Esch. coli* are more frequent among women than among men, 18 male and 18 female »healthy» medical students of approximately the same age were examined. The mean age for the women was 25 years and for the men 22 years.

TABLE 13

CORRELATION OF ACL VALUES AND SEX IN »NORMAL» SUBJECTS

Sex	No. of Subjects	Mean Age	ACL $<$ 400	ACL $\geqq$ 400	ACL $>$ 400
Female	18	25	17	1	1
Male	18	22	16	1	2

In the light of the results in Table 13, there is no definite difference between the incidence of elevated ACL values for »healthy» males and »healthy» females in, at least, this age group.

3. MEAN ERROR OF THE ANTICOLILYSIN REACTION

To ascertain the mean error in the anticolilysin reaction, 50 »healthy» and pathological sera selected at random were titrated twice in conjunction with other titrations and without identification of these sera.

The mean error of the anticolilysin reaction was calculated on the basis of the two titrations according to the following formula:

$$\frac{1}{2}\sqrt{\frac{\Sigma\,(x_I - x_{II})^2}{50}}$$

where x_I and x_{II} are the logarithms of the titers obtained at the two readings. This calculation gave a mean error of $\pm$ 15 per cent.

Fig. 9. — Diagram showing distribution of 206 »normal» sera by age of patients and anticolilysin titers (ACL).

4. SPECIFICITY OF THE ANTICOLILYSIN REACTION

The routine examinations of patient sera were made with colilysin from which the bacteria had been removed by high speed centrifugation. A parallel titration of 20 random sera was therefore carried out with colilysin from the some strain, purified by precipitation with methyl alcohol. When calculated according to the formula given above, a mean error of $\pm$ 17 per cent was obtained. Since this result is well in harmony with the mean error obtained above, the results of the titrations with centrifuged colilysin may be regarded as dependable. All titrations of »normal» and pathological sera in this investigation were therefore performed with centrifuged peptone water-lysin from one strain (No. 24).

The specificity of the anticolilysin reaction in relation to other antihemolysin reactions was controlled by titration with colilysin of 100 sera from routine titrations of AST and Asta reactions. The correlation between the positive and negative anticolilysin values and the corresponding AST and Asta values was examined. To obtain a larger material for comparison, the ACL borderline value of 400 was included among the elevated titers.

TABLE 14

CORRELATION OF POSITIVE AND NEGATIVE ANTICOLI REACTIONS WITH AST AND ASTA REACTIONS IN 100 SERA

	AST $<$ 200		AST $>$ 200		Asta $<$ 2		Asta $>$ 2	
	%	No.	%	No.	%	No.	%	No.
ACL $\geqq$ 400	33 %	22	24 %	8	30 %	24	30 %	6
ACL $<$ 400	67 %	45	76 %	25	70 %	56	70 %	14

Table 14 shows that there was no correlation between the AST and Asta reactions and the ACL reaction. Since in another test no inhibition of hemolysis was obtained when standard AST and Asta sera with a high antihemolytic effect were titrated with colilysin, the ACL reaction may probably be regarded as specific for anticolilysin and fully independent of the analogous AST and Asta reactions.

C. ANTICOLILYSIN IN PATIENT SERA

1. REVIEW OF THE LITERATURE ON INCIDENCE OF *ESCH. COLI* IN VARIOUS INFECTIOUS DISEASES

Infections of the Urinary Tract. — Numerous studies point to the high incidence of *Bact. coli* as the infective agent in urinary tract infections. Reports by Meyer & Löwenberg (19), Bitter & Gundel (2) and Vahlne (31) have already been referred to in Chapter I (page 9).

In a series of 426 cases, Side & Luz (28) found *Bact. coli* in 71 per cent of cases of pyelitis and 64 per cent of cystitis.

Dudgeon, Wordley & Bawtree (4) examined 27 strains of *Bact. coli* isolated from urinary tract infections in male patients and demonstrated that 20 strains were hemolytic. A similar examination of 42 female patients showed hemolytic activity by 11 strains.

Gallbladder Infections. — Clinically, a large proportion of cases of cholecystitis occur in association with gallstones, and opinions differ greatly regarding the process which should be regarded as primary.

Fuld (6) made a compilation of the results obtained in a number of bacteriological investigations on the occurrence of various bacterial agents in acute cholecystitis. This statistical material, covering the period 1892—1927, revealed that 56 per cent of the cases had given cultures of *Bact. coli commune.*

Vahlne (31), in his bacteriological examination in a number of operated cases of gallbladder diseases, was able to demonstrate *Bact. coli* chiefly in cases of empyema of the gallbladder.

In a bacteriological investigation of 325 cases operated on for diseases of the gallbladder, H. Renkonen (23) found the majority of the bacteria to be *Esch. coli*, which were present in 23 per cent of the cases, *i.e.*, in pure cultures in 63 cases and in mixed cultures in 13 cases. The incidence of *Esch. coli* cultures increased in elderly persons, being in the age group of 60—69 years 39 per cent and in the group of 70—79 years 100 per cent of the cases, as seen in Table 15. It is also of interest that 65 per cent of the bacterial cultures in the age groups over 50 years contained *Esch. coli.*

TABLE 15

INCIDENCE OF POSITIVE BACTERIAL CULTURES FROM EXCISED GALLBLADDERS, BY AGE OF PATIENTS (ACCORDING TO H. RENKONEN)

Age Group	No. of Cases	Positive Bacterial Cultures		Occurrence of *Esch. coli*	
		No.	%	No.	%
10—19	2	0	0 %	0	0 %
20—29	32	5	16 %	0	0 %
30—39	60	21	35 %	8	13 %
40—49	92	36	39 %	17	18 %
50—59	76	37	49 %	22	29 %
60—69	56	35	63 %	22	39 %
70—79	7	7	100 %	7	100 %
Total	325	141	43 %	76	23 %

Infectious Gynecological Diseases. — In bacterial cultures from vaginal secretion in subfebrile cases following parturition or abortion, Schottmüller (25) demonstrated the presence of *Bact. coli* in c. 23 per cent. Warnekros (32) found a high incidence of this bacterium in blood cultures made immediately after evacuation of the uterus in cases of febrile abortion with chills. This worker suggested that lesions in the uterine wall permit the entry of *Bact. coli* into the blood stream.

Schottmüller & Barfurth (26) examined samples of pus from inflammatory tumors of the adnexa and pelvic peritonitis and found *Bact. coli* in 10 per cent of the cases.

In a series of 101 cases of acute salpingitis, Holz (9) demonstrated *Bact. coli* in 5 cases, and in 1 case only in a series of 39 chronic cases. No bacterial growth was obtained in 42 acute cases. In the remaining cases the cultures revealed mainly streptococci and staphylococci.

Bucura (3), as well as several other investigators, regards streptococci as the most frequently encountered pathogenic organism in inflammatory adnexitis. Among other infective agents he also mentions staphylococci and *Bact. coli.*

In examining 25 cases of chronic tubo-ovarian abscess, Attemeyer (1) found *Bact. coli* in 2 cases and anaerobic streptococci in 22 cases. The mean age of the patients in this series was 35.5 years.

Heynemann (8), in 150 punctures of pyosalpinx, demonstrated *Bact. coli* in 13 cases, anaerobic streptococci in 38 cases, staphylococci in 19 cases, and hemolytic streptococci in 8 cases. No cultures of bacteria were obtained in 82 cases and the findings in the remaining cases were referable to different bacterial groups.

Schröder (27) reports that four-fifths of the inflammatory processes in the adnexa are encountered in patients under 35 years of age, and that gonococci and abrasions followed by secondary infection should be regarded as the principal causes.

In an earlier paper (34) I have reported the results of Asta reactions in the sera of 90 patients with acute and chronic gynecological infections. Elevated Asta values were obtained in 48 per cent of the chronic infections, whereas the acute cases showed nearly normal values.

Infantile Enteritis and Gastro-Enteritis. — Among other workers, Kauffmann & Dupont (12) have assigned the strains of *Esch. coli* isolated from infantile diarrhea chiefly to O groups 55 and 111.

Appendicitis. — A number of opinions have been advanced on the etiology of appendicitis, and the part played by *Esch. coli* has been frequently discussed in this connection. Thus, *e.g.*, Warren (33) and Löhr (17) demonstrated a high incidence of *Bact. coli* in operated appendices examined by them.

Kauffmann (10), in examining operated appendices, encountered serologically differentiable types of *Esch. coli* possessing a higher pathogenicity than the strains in the normal fecal flora. He was therefore of the opinion that the principal etiological agents in appendicitis and appendix peritonitis are strains of *Esch. coli* belonging to these special serological groups.

In the bacteriological examination of 583 appendices, Vahlne (31) obtained cultures of *Bact. coli* in all but 48 cases.

2. OWN INVESTIGATIONS

a. Anticolilysin (ACL) in Infections of the Urinary Tract Caused by *Esch. coli*

Sera from 28 patients with infections of the urinary tract were examined. In all of these cases *Esch. coli* was cultivated from the sterile urine. The cases were classified into two groups according

TABLE 16

DISTRIBUTION OF ACL TITERS IN PATIENTS WITH DIAGNOSED *Esch. coli* INFECTIONS OF THE URINARY TRACT

Diagnosis	No. of Cases	Mean Age	A C L										
			50	100	150	200	300	400	800	1600	3200	>400	≧400
Pyelocystitis ..	14	46 yr.	—	—	1	3	1	3	3	—	3	43%	64%
Hypertrophy of the prostate with cystitis	14	61 »	2	4	1	3	1	1	—	2	—	14%	21%
Total	28	53.5 yr.	2	4	2	6	2	4	3	2	3	29%	43%

to diagnosis, as follows: 1) *Pyelocystitis*, in which the urinary sediment contained, in addition to *Esch. coli*, numerous leukocytes, erythrocytes and epithelial cells, and 2) *Hypertrophy of the prostate with cystitis*, which were afebrile cases with no clinical symptoms of pyelitis.

The ACL values obtained in titrations of the sera of these patients are shown in Table 16. It is noted that in group 1, which consists of cases of pyelocystitis, elevated titers (ACL > 400) were present in 43 per cent of the cases. However, if a titer of 400 is also regarded as elevated, 64 per cent of the cases in this group showed elevated values (ACL $\geqq 400$). On the other hand, in group 2, comprising cases of hypertrophy of the prostate with cystitis, only 14 per cent of the cases had values of > 400 and 21 per cent values of $\geqq 400$, as compared with 8 per cent and 20 per cent in the »normal» series. As the mean age of the patients in this group was 61 years and as at this age there is an upward trend in the ACL curve for the »normal» series, the incidence of elevated ACL titers obtained for this group of patients seems to indicate that there is no significant production of anticolilysin in these cases. The reverse is the case with the pyelocystitis group, in which the incidence of ACL > 400, as said, was 43 per cent. It may be mentioned that the 3 patients in this group who had a titer of 3200 were chronic cases in which the elevated values persisted regardless of extended antibiotic therapy.

An example of the possibilities of clinical application of the ACL reaction is offered by a case which was under observation after this investigation was completed. This was a male patient 78 years

of age, who 2 years previously had undergone operation for hypertrophy of the prostate. On December 17, 1952, the culture of bacteria from catheterized urine showed a mixed infection with *Esch. coli*, *Proteus* and *Streptococci*, and the urinary sediment contained numerous leukocytes and erythrocytes. At this time the SR was 22 mm./1 hr. and the ACL = 400. Regardless of antibiotic therapy based on bacterial resistance tests, the titer was 10 days later ACL = 800 and 14 days later ACL > 1600. AST titrations made at the same time gave the low values of 40, 36 and 32. In the meantime, following cystostomy and extraction of calculi, there was a marked decline in the patient's condition and he died of chronic pyelocystitis and mild uremia. It seems justified to conclude that the clinical course of the case was reflected in the increasing ACL values.

b. ACL in Diseases of the Gallbladder

The sera of 73 patients with diseases of the gallbladder were tested in this series, in most cases before cholecystectomy. The patients were classified into 3 groups, as follows: 1) *Cholecystitis or cholangitis*, 2) *Cholelithiasis*, and 3) *»Cholecystopathy» or »dyskinesia of the gallbladder»*. The diagnoses were confirmed mainly at roentgenological examination and surgical operation.

Group 3 consisted of operated and non-operated cases in which the clinical findings did not give reason for inclusion in groups 1 or 2. However, it should be noted that 40 per cent of the patients in group 3 had a sedimentation rate of over 20 mm./1 hr. and that the average rate for the group was 20 mm./1 hr.

None of the patients in the series of gallbladder diseases had a case history of less than 2—3 months.

Table 17 shows the ACL values obtained by titration of the sera of these patients. In the cholecystitis group, increased values (ACL > 400) were seen in 48 per cent of the cases, or if the titer of 400 is also regarded as elevated, in 73 per cent. In consideration of the fact that the »normal» series in this investigation gave values of > 400 in 8 per cent and of ≧ 400 in 20 per cent, there was a very considerable increase in the ACL in the cases with cholecystitis or cholangitis.

TABLE 17

DISTRIBUTION OF ACL VALUES IN PATIENTS WITH DISEASES OF THE GALLBLADDER

Diagnosis	No. of Cases	Mean Age	SR	A C L												
				50	100	150	200	300	400	600	800	1200	1600	3200	>400	≧400
Cholecystitis or cholangitis	23	53 yr.	80 % >20 mm./1 hr. Mean 44 mm./1 hr.	—	—	—	4	2	6	2	4	3	2	—	48 %	73 %
Cholelithiasis	30	49 »	43 % >20 mm./1 hr. Mean 27 mm./1 hr.	—	2	1	7	2	12	1	5	—	—	—	20 %	60 %
»Cholecystopathy» or »dyskinesia of gallbladder»	20	49 »	40 % >20 mm./1 hr. Mean 20 mm./1 hr.	1	2	—	8	3	1	—	3	—	1	1	25 %	30 %
Total	73	50 yr.	—	1	4	1	19	7	19	3	12	3	3	1	30 %	56 %

Elevated values were seen in the cholelithiasis group as follows: > 400 in 20 per cent and $\geqq 400$ in 60 per cent of cases. Thus these patients also showed elevated values, although to a smaller extent than the subjects in the preceding group.

In the »cholecystopathy» group, titers of > 400 were seen in 25 per cent and of $\geqq 400$ in 30 per cent. This finding and the relatively high sedimentation rates, referred to above, may be ascribable to a possibly incomplete differential diagnosis of a previous inflammation of the gallbladder.

The mean ages of the patients in the 3 groups in this series were uniform and the groups may therefore be regarded as comparable. As was to be expected, the highest SR values were seen in the cholecystitis group, which showed rates of over 20 mm./1 hr. in 80 per cent of the cases and a mean SR of 44 mm./1 hr. for the group. In the other two groups of patients, the SR was over 20 mm./1 hr. in c. 40 per cent of the cases.

When comparing the cholecystitis group in the present investigation with the series reported by Fuld (6), in which *Bact. coli commune* was diagnosed in 56 per cent of the cases, it is noted that the incidence of 48 per cent (73 per cent) of elevated ACL values in my cholecystitis group agrees well with Fuld's finding.

The incidence of elevated ACL titers obtained in this series also bears out the observation by H. Renkonen (23) of a high incidence of *Esch. coli* in cholecystectomized cases of gallbladder disease.

c. ACL in Infectious Gynecological Diseases

In this series the sera of 82 patients were examined. These cases of infectious gynecological diseases were divided into 3 groups according to the clinical finding and the duration of the disease. Group 1 consisted of cases of febrile abortion or of post-abortion infection. Group 2 included acute infections diagnosed as acute salpingo-oophoritis, pyosalpinx or abscess of the pouch of Douglas. In view of the relatively long time required for the production of antibodies, no cases with a history of less than 3 weeks were included. Group 3 comprised cases of chronic infection and included the diagnoses of chronic salpingo-oophoritis, parametritis, pyosalpinx and pyo-ovarium.

TABLE 18

DISTRIBUTION OF ACL TITERS IN PATIENTS WITH INFECTIOUS GYNECOLOGICAL DISEASES

Diagnosis	No. of Cases	Mean Age	SR	A C L														
				50	100	150	200	250	300	400	600	800	1200	1600	2400	3200	>400	≧400
Febrile abortion or post-abortion infection ..	20	30 yr.	80%>20 mm./1 hr. Mean 48 mm./1 hr.	—	1	1	4	—	4	4	—	6	—	—	—	—	30%	50%
Acute gynecological infections	23	37 »	82%>20 mm./1 hr. Mean 56 mm./1 hr.	1	2	2	8	—	2	5	—	2	—	—	—	1	13%	35%
Chronic gynecological infections	39	34 »	82%>20 mm./1 hr. Mean 50 mm./1 hr.	—	2	2	6	1	3	9	4	8	1	2	1	—	41%	64%
Total	82	34 yr.	—	1	5	5	18	1	9	18	4	16	1	2	1	1	30%	52%

As in the series of gallbladder diseases, the cases in the different groups were distributed according to the ACL, SR and age of patients. This distribution is shown in Table 18.

It will be noted that 30 per cent of the 20 cases of infective abortion (group 1) had an ACL of > 400, 50 per cent of $\geqq 400$, and 80 per cent a SR of over 20 mm./hr. In group 2, consisting of 23 cases of acute infection, ACL values of > 400 were only seen in 13 per cent and $\geqq 400$ in 35 per cent. In 82 per cent the SR was over 20 mm./1 hr. Group 3, comprising 39 cases of chronic infection, gave ACL values of > 400 in 41 per cent and $\geqq 400$ in 64 per cent. In 82 per cent of these cases the SR was over 20 mm./1 hr.

In examining the results for the different groups in this series, no correlation is seen between elevated SR and elevated ACL values, as is clearly evident from the acute cases of infection in group 2. The SR was over 20 mm./1 hr. in 82 per cent of these cases, and the mean SR for the group 56 mm./1 hr. However, the distribution of ACL values in this group is nearly similar to that of the »normal» series.

In the cases of abortion in group 1, and especially in the chronic infections in group 3, the incidence of elevated ACL titers was high. The blood cultures made in cases of febrile abortion by Warnekros (32) gave a high incidence of *Esch. coli*, and the results of the ACL determinations in the present investigation harmonize with this statement.

Although some workers have reported a low incidence of *Esch. coli* in infectious gynecological diseases, the results obtained by the ACL reaction seem to indicate that the *Esch. coli* should probably be ascribed a significant rôle as a causative agent in secondary infections superimposed on abortion, as well as in chronic gynecological infections.

d. ACL in Infantile Enteritis and Gastro-Enteritis

The importance assigned by some workers in recent years to *Esch. coli* in the etiology of infantile diarrhea prompted the examination of the ACL reaction in a series of 31 cases of acute enteritis and gastro-enteritis in children. Symptoms of intoxication were associated in some cases.

As the production of antibodies in children is not comparable with the reaction in adults, 20 »healthy» children of the same age were used as controls.

The ACL values obtained for the »normal» and patient sera are shown in Table 19. They reveal no increased incidence of

TABLE 19

DISTRIBUTION OF ACL TITERS IN »HEALTHY» CHILDREN AND CHILDREN WITH ENTERITIS OR GASTRO-ENTERITIS

Diagnosis	No. of Cases	Mean Age	ACL								
			50	100	150	200	300	400	800	1600	≧400 (%)
»Healthy»	20	6 mth.	12	1	1	1	1	4	—	—	20.0 %
Acute enteritis or gastro-enteritis ..	31	8 »	20	6	—	3	—	1	—	1	6.4 %

elevated ACL values in the children with enteritis or gastro-enteritis. However, one of the reasons may possibly be the low mean age of 8 months and the consequently poor formation of antibodies. In case infantile gastro-enteritis is a condition similar to bacillary dysentery, no significant production of antibodies probably takes place. A number of reports in the literature on the low antibody production in dysentery mention negative and relatively low antidysentery titers in the presence of positive bacterial findings. Thus, *e.g.*, Floch (5) obtained positive serum agglutination in less than 10 per cent of c. 3,000 patients with Shiga or Flexner dysentery. Similar observations have also been reported by Kolle & Hetsch (16) and Löhr (18).

Furthermore, the pathogenic rôle of *Esch. coli* in infantile diarrhea has probably been overestimated by some authors.

e. ACL in Various Other Diseases

In this series, a case of colisepticemia was examined. This was a female patient, 53 years of age, who had been under medical care for some time following splenectomy performed 2 years previously. The patient was now hospitalized in a febrile and generally poor condition with the diagnosis of *Septicaemia Esch. coli.*

Status postsplenectomiam. Anaemia normochromica. The sedimentation rate was 70 mm./1 hr. and *Esch. coli* were found in 3 blood cultures made on successive days. At this time there was a septic fever curve, with the temperature *ad* 41° C. The ACL was 6400. In view of the high titer, the finding of *Esch. coli*, and the probable absence of factors with a non-specific effect on the ACL, and as the AST value was 150 and the Asta value 2, a serum sample was taken from this patient for use as a standard serum in these investigations (cf. page 44).

This case can therefore be regarded as a clearly evident example of the correlation between the ACL and a confirmed infection with *Esch. coli.*

ACL values that were within normal limits were obtained in 8 operated cases of gangrenous appendicitis, 2 of which also showed symptoms of irritation of the peritoneum.

On the other hand, titrations in 3 cases of pancreatitis gave ACL values of 400, 800 and 1600, respectively.

A similar distribution of ACL values was seen in titrations of the sera from 3 patients with acute nephritis with no signs of past or present colibacillary infection. The ACL values obtained in these cases were 1200, 3200 and 3200, respectively.

As the number of patients in these 3 groups was small and as in the last 2 groups there may have been changes in the serum fractions, I have refrained from drawing any conclusions from the results in this series of cases, with the exception of the individual case of *Esch. coli* septicemia, which showed a striking correlation between the disease condition, bacterial finding and the ACL titer.

D. SUMMARY.

Rabbits were immunized with lysins prepared from *Esch. coli* strains of O groups 2 and 54. The course of immunization was followed by titrations of serum at regular intervals. After an immunization period of 50 days it was possible to obtain serum with a good antihemolytic effect. When the injections of colilysin were discontinued the antihemolytic activity of the serum declined steadily. All the samples taken during the immunization period were titrated with homologous and heterologous colilysin. The

results of the two titrations were concordant. Control titrations with streptolysin and staphylolysin were made with the immune sera used as standard sera, but no antihemolytic activity could be demonstrated.

A method for determination of the anticolilysin titer (ACL), analogous to the antipneumolysin reaction, was developed.

The ACL of the sera of 206 »healthy» persons of different age groups was determined. The sera of 8 per cent of these »healthy» persons showed titers of over 400 (ACL $>$ 400) and 20 per cent titers of 400 or over (ACL $\geqq$ 400). A titer of 400 has therefore been regarded as moderately elevated and titers over 400 as elevated. It is noteworthy that the curve for the mean ACL values of all the age groups rises in the elderly groups.

To study the possible difference between the sexes, 18 male and 18 female »healthy» medical students of the same age group were examined. No noteworthy difference was seen in the distribution of the ACL in the two groups.

The standard error of the anticolilysin reaction was calculated to be $\pm$ 15 per cent on the basis of titers obtained for 50 sera titrated twice in succession.

Parallel titrations of 20 random sera were made with the usual centrifuged peptone water-colilysin and with colilysin from the same strain purified by precipitation with methyl alcohol. The results of the two titrations were in agreement with each other and the standard error was $\pm$ 17 per cent. In another test, parallel titrations of 100 random sera were made with streptolysin, staphylolysin and colilysin. No correlation was seen between the AST and Asta reactions and the ACL reaction. In a further test no inhibition of hemolysis was obtained when standard AST and Asta sera with a high antihemolytic effect were titrated with colilysin. The anticolilysin reaction may probably be regarded as specific for anticolilysin and fully independent of the analogous AST and Asta reactions.

In the examination of sera from 28 patients with a finding of *Esch. coli* in the urine, a group consisting of 14 cases of pyelocystitis showed values of ACL $>$ 400 in 43 per cent and of $\geqq$ 400 in 64 per cent. In a group of 14 cases of hypertrophy of the prostate with cystitis, titers of $>$ 400 were obtained in 14 per cent only and of $\geq$ 400 in 21 per cent. Outside this series there was a fatal case of

chronic pyelocystitis with findings of *Esch. coli*, *Proteus* and *Streptococci*, in which rising ACL values were seen as the condition aggravated. The AST values obtained in parallel tests were constantly low.

ACL determinations were carried out with the sera of 73 patients with various diseases of the gallbladder. Of 23 patients with cholecystitis or cholangitis, 48 per cent gave ACL > 400 and 73 per cent ACL $\geqq 400$. SR values over 20 mm./1 hr. were seen in 80 per cent of the cholecystitis and cholangitis cases. In the group of 30 cases of cholelithiasis the ACT was > 400 in 20 per cent and $\geqq 400$ in 60 per cent, and 43 per cent of the cases had a SR of over 20 mm./1 hr. The remaining 20 patients in the series of diseases of the gallbladder, given the clinical diagnosis of »cholecystopathy» or »dyskinesia of the gallbladder», gave values of ACL > 400 in 25 per cent and $\geqq 400$ in 30 per cent. In 40 per cent the SR was over 20 mm./1 hr.

The sera of 82 patients with infectious gynecological diseases were examined in three groups. In the first group of 20 febrile abortions or post-abortion infections, titers of >400 were obtained in 30 per cent and $\geqq 400$ in 50 per cent of the cases. In 80 per cent the SR was over 20 mm./1 hr. The group of 23 acute infections (adnexitis, etc.), showed nearly normal values, *i.e.*, ACL > 400 in 13 per cent and ACL $\geqq 400$ in 35 per cent, whereas the SR was over 20 mm./1 hr. in 82 per cent of the cases. In the series of chronic infections, comprising 39 cases, 41 per cent gave ACL > 400 and 64 per cent $\geqq 400$. The SR in this group was over 20 mm./1 hr. in 82 per cent, also.

The sera from 31 children (mean age 8 months) with enteritis and gastro-enteritis showed no elevated ACL values when compared with a corresponding series of 20 »healthy» children of the same mean age.

Negative results were also obtained in ACL determinations of the sera of 8 patients operated on for gangrenous appendicitis. On the other hand, elevated ACL titers were seen in 3 cases of pancreatitis and 3 cases of acute nephritis.

The highest ACL titer (6400) was found in a case of colisepticemia, in which the AST reaction was at the same time 150 and the Asta reaction 2.

CHAPTER IV

DISCUSSION

After Kayser (13) reported in 1903 the occurrence of a heat-resistant, filtrable hemolysin produced by *Esch. coli*, this subject was given relatively little attention for a long time. The major reason was probably the failure of other investigators to repeat Kayser's experiment. It was assumed by Robinson (24) that the hemolytic reaction obtained by Kayser was not produced by a lysin but by carbonates developed during bacterial growth.

Meanwhile, a number of reports have been published on the incidence and serological grouping of hemolytic strains of *Esch. coli* and on their pathogenicity. It has been suggested by some investigators that a more acute disease condition is produced by the hemolytic than the non-hemolytic strains of *Esch. coli*.

The selection of the strains of *Esch. coli* for this investigation and the serological typing of the hemolytic strains are described in Chapter I. Horse red cells have commonly been used as indicators of the hemolytic activity of *Esch. coli*, but since preliminary tests, described in Chapter II, showed that sheep red cells were more sensitive to colilysin, a 2 per cent suspension of these cells was used in all the tests in this investigation. Chapter II describes the different experiments made for the production of colilysin under various conditions, and the observations and conclusions made in regard to the nature and properties of this lysin.

At an early stage of this investigation the great lability of the colilysin became evident, and this was the chief difficulty experienced throughout the work. It was also seen that the principles followed in the production of other bacterial lysins could not be

applied in this connection. Colilysin was produced by culture of hemolytic strains of *Esch. coli* in different culture media, *i.e.*, ordinary beef broth, various modifications of 1 per cent peptone water and of casein hydrolysate solution, and at a later stage in a medium defined by Robinson (24).

The production or the stability of the colilysin were not influenced demonstrably if the bacteria were grown in O_2 or N_2 atmospheres, nor was the lysin stabilized by adding to the cultures, after growth, various reducing substances, such as sodium thioglycolate, cysteine, ascorbic acid and sodium hydrosulphite. Thus it could not be demonstrated that the lability of colilysin would be due to oxidation, as is the case when streptolysin O, for instance, is produced. On the other hand, some correlation was seen during colilysin production between the declining pH of the culture medium and the diminishing activity of the colilysin. However, no definite indications were obtained that the pH would be the only factor responsible for the inactivation.

A marked difference was seen in the stability of lysins produced in 1 per cent peptone water with 0.5 per cent glucose added and in Robinson's medium. The activity of the colilysin began to decline sharply after 5 to 6 hours' growth of bacteria in peptone water, associated with a definitely declining pH, whereas the lysin produced in Robinson's medium still retained its activity after 48 hours' growth, in association with a fairly constant and sometimes even rising pH. It was not possible to re-activate inactive peptone water-lysin by increasing the pH of the medium. Thus the inactivation of colilysin is probably an irreversible reaction. Attempts to demonstrate the presence of some inactivating factor in completely inactivated colilysin were unsuccessful.

As was reported by Robinson, colilysin is thermolabile. In the present experiments the lysin became inactive within 1 hour at +56° C. but retained its hemolytic activity for about 20 hours at +37° C.

Sjöstedt (29) and Robinson (24) state that they did not succeed in repeating Kayser's experiment and separating the lysin from the bacteria by filtration. My attempts to do so were also unsuccessful. However, high speed centrifugation yielded a clear, active colilysin from the peptone water and casein hydrolysate media but not from Robinson's medium. In the latter case the lysin was totally

inactivated during centrifugation. The titrations of patient sera were therefore chiefly carried out with centrifuged peptone water-lysin, even at the risk of more rapid inactivation of the colilysin.

In an effort to obtain a purer and possibly a more stabile colilysin, centrifuged casein hydrolysate-colilysin was precipitated with methyl alcohol at —10° C. An active lysin was obtained but it was difficult to dissolve it in H_2O, physiological saline or N./10 Sörensen's phosphate buffer solution at varying pH levels. Furthermore, the lability of the purified lysin was equal to that of the colilysin before precipitation. When precipitation was carried out with a saturated solution of $(NH_4)_2\ SO_4$ at $\pm 0°$ C. a similar quantitative yield was obtained but the precipitate was very readily inactivated during dialysis. No hemolytic activity was obtained in control precipitations of the culture media alone.

An active colilysin in powdered form was obtained when the colilysin separated by precipitation with methyl alcohol was lyophilized. However, when stored at room temperature the powdered lysin possessed no greater stability than before.

A number of experiments were made to find a method for the storage of colilysin. However, although streptolysin and staphylolysin will retain their hemolytic power for about 3 months if stored in sterile flasks at +4° C., the colilysin under similar conditions began to show reduction of activity already after 24 hours. At —18° C. the storage time could be extended to 1 week with practically no change in the hemolytic activity. The addition of a 25 per cent sterile solution of glycerin increased the storage time at —18° C. to 21 days with fairly well retained activity. The addition of reducing substances that are used in the storage of streptolysin appeared to have no stabilizing effect on the colilysin. Lysin produced in casein hydrolysate, precipitated with methyl alcohol and lyophilized retained its activity for 8 months when stored in vacuum-sealed ampoules at —18° C.

It has been seen that the high lability and the irreversible inactivation of the colilysin formed a problem in all the experiments described in Chapter II. However, the successful storage of the powdered colilysin for a period of 8 months would seem to be a result worth noting. In my opinion further experiments should be made to purify colilysin in order to perfect a method of storage, since purity, obtained in my experiments by precipitation, made

a longer period of storage possible. A purer lysin would also enable us to study the nature of colilysin, which did not now prove possible.

Preparatory to study of the production of anticolilysin in human serum, the antigenic properties of colilysin were studied in animal experiments, which are described in Chapter III. The immunization experiments with rabbits, using colilysin produced from two different strains of *Esch. coli*, revealed that a powerful production of anticolilysin took place in the serum of the immunized animals. After discontinuation of the colilysin injections the anticolilysin content of the sera showed a steady decline.

The course of the anticolilysin production during the immunization period was followed by serological antihemolysin tests. When at the end of the immunization period of 50 days all the sera withdrawn and stored during the experiment were titrated with homologous and heterologous colilysin, concordant values were obtained with the two lysins. Rabbit immune sera with high anticolilysin titers were also titrated with streptolysin and staphylolysin but no inhibition of hemolysis was seen. Thus colilysin probably is a specific whole antigen.

In analogy with the antistreptolysin (AST), antistaphylolysin (Asta) and antipneumolysin (APL) reactions, evaluations of the anticolilysin titer (ACL) of human sera were made. In determining the borderline between normal and elevated anticolilysin titers it was regarded that all values below 400 were normal, a value of 400 moderately elevated, and values above 400 elevated. This classification was based on the findings in a series of 206 »healthy» persons of all age groups, in whose sera ACL titers of more than 400 (ACL > 400) were obtained in 8 per cent and titers of 400 or over (ACL $\geqq 400$) in 20 per cent. A noteworthy feature is the rise in the ACL curve after the age of 45 years (Fig. 9). This should probably be ascribed to an increasing incidence of infections by *Esch. coli* in the elderly age groups (23). No difference in the distribution of the ACL values in »healthy» males and females was found in a small series of 36 »healthy» medical students of the average ages of 22 and 25 years, respectively.

The standard error of the anticolilysin reaction was calculated on the basis of the logarithms of the titers obtained in two successive titrations of 50 random sera. The standard error was found to

be ±15 per cent, which is in agreement with the other hemolysin reactions.

As the bacterial cells could not be removed from the culture medium by filtration, the dependability of the centrifuged peptone water-colilysin used in the routine titrations of patient sera was controlled by the parallel titration of 20 random sera with centrifuged colilysin and with alcohol-precipitated, purified colilysin from the same strain of *Esch. coli*. The results of the two titrations were well in harmony and the standard error was ±17 per cent.

As already said, the anticolilysin reaction appears to be a specific reaction, at least in so far as the AST and Asta reactions are concerned. No correlation was seen between the results of the ACL reaction and the AST and Asta reactions in parallel titrations of 100 random sera. Furthermore, no inhibition of hemolysis could be demonstrated when AST and Asta standard sera were titrated with colilysin. Neill, Fleming & Gaspari (20) and Oker-Blom, Nikkilä & Kalaja (22) have shown that the inhibition of hemolysis cannot always be ascribed to the presence of an antihemolysin, since in certain cases it may be the result of changes in the serum lipid fraction. In the present investigation no serum cholesterol tests were made. It may indeed be possible that sources of error of this kind may occur in individual cases, thus also explaining some unexpectedly high ACL values.

In titrations of the sera of 28 patients with bacteriological findings of *Esch. coli* in the urine, greatly elevated ACL values were obtained for the patients with pyelocystitis. Titers up to 3200 were seen in the chronic cases. However, no marked increase was present in cases of hypertrophy of the prostate associated with more or less latent cystitis. The results in these cases and particularly in the reported case of chronic infection of the urinary tract with *Esch. coli*, in which latter progressively rising ACL values were obtained, are evidence supporting the opinion that the ACL reaction reflects the course of a deep-lying infection with *Esch. coli*. It should therefore be possible to draw certain conclusions from the incidence of elevated ACL values, especially in disease conditions in which a bacteriological examination cannot be made.

In view of the common occurrence of *Esch. coli* in diseases of the gallbladder, determination of the ACL was made in 73 cases of these diseases. In the cholecystitis group the incidence of elevated titers

was considerably higher than in the »normal» series. We may be justified in considering that this reflects the significant rôle assigned in the literature to *Esch. coli* in cholecystitis. The cholelithiasis group also showed a higher incidence of elevated ACL values than the »normal» series, whereas the »cholecystopathy» group did not give as high values, if the moderately elevated cases are included. It therefore seems apparent that the ACL reaction can be given a certain degree of importance as a clinical aid in the diagnosis of diseases of the gallbladder, provided, of course, that the presence of other colibacillary infections can be excluded.

The structure and anatomical location of the female genital organs in relation to the intestinal tract and the possibilities consequently present for secondary infection with, for instance, *Esch. coli*, prompted an ACL examination also in a series of patients with infectious gynecological diseases.

In this series of 82 cases the highest incidence of elevated titers was obtained in the group of chronic infections, which included the diagnoses of chronic salpingo-oophoritis, pyosalpinx, pyo-ovarium and parametritis. The distribution of the ACL values (> 400 in 41 per cent and $\geqq 400$ in 64 per cent) speaks for a secondary infection with *Esch. coli* in a large number of patients in this group. Definitely elevated titers were also obtained in the group consisting of febrile abortions and post-abortion infections. This is well in agreement with the results of the bacterial examinations made by Warnekros (32) in infectious cases of abortion, which showed a high incidence of *Esch. coli*. On the other hand, the group of other acute gynecological infections, consisting of acute salpingo-oophoritis, pyosalpinx and abscess of the pouch of Douglas did not reveal a very high incidence of elevated ACL values.

In consideration of the low incidences of *Esch. coli* reported by some authors in bacteriologically examined cases of infectious gynecological diseases, the high ACL values obtained in this investigation, especially in the chronic cases, are interesting. Provided that the presence of other infections produced by *Esch. coli* can be excluded, the ACL reaction should probably be of some clinical importance in the selection of antibiotic therapy in gynecological infections.

In a children's series consisting of 31 cases of enteritis and gastroenteritis, no definitely elevated titers were seen. This may possibly

be ascribed to the low mean age of the children (8 months) and to the more or less acute character of the disease. If infantile enteritis is a condition similar to bacillary dysentery it probably does not cause significant production of antibodies. Furthermore, the pathogenic rôle of *Esch. coli* in infantile diarrhea has probably been overestimated by some authors.

The very high ACL value of 6400, appearing concurrently with an AST titer of only 150 and an Asta titer of 2 in a bacteriologically confirmed case of septicemia caused by *Esch. coli* may be regarded as an example of the correlation of the ACL and the severity of the disease process.

Regardless of the numerous reports in the literature ascribing an important rôle to *Esch. coli* in appendicitis, elevated titers were not seen in this group. This, however, may be due to the small number of cases studied. The effective antibiotic treatment now given in combination with more rapid surgical intervention apparently also decreases the formation of antibodies.

Results that may be considered interesting were obtained in 3 cases of pancreatitis, as in all of these the ACL was elevated. Considerably higher titers were obtained in 3 cases of acute nephritis, with no signs of past or present infection with *Esch. coli*. However in view of the small number of cases in these two groups and of the possibility of changes in the serum fractions, the drawing of definite conclusions from these findings would probably not be justified.

From the results which were obtained in the tests made in this investigation it would appear that the anticolilysin reaction probably has a certain clinical importance. A statistical analysis of the results of anticolilysin titrations may provide significant information for the evaluation of the part played by *Esch. coli* in various diseases.

CHAPTER V

CONCLUSIONS

1) Out of 200 routine cultures of bacteria, 130 strains were diagnosed as *Esch. coli* on the basis of bacteriological tests. The hemolytic power of these strains was tested against a 2 per cent suspension of sheep red cells, which were found to be the most suitable indicators of hemolytic activity in these tests. Of the total of 130 strains, 47 (36 per cent) were hemolytic. Twelve strains with the highest hemolytic power were referred serologically to different O groups.

2) Colilysin was produced by culture of hemolytic strains of *Esch. coli* in ordinary beef broth and in different modifications of 1 per cent Witte's peptone water, casein hydrolysate solution, and a medium defined by Robinson. The substrates chosen for the continued experiments were 1 per cent Witte's peptone water with 0.5 per cent glucose added, and casein hydrolysate solution with 0.5 per cent glucose.

3) Colilysin is a thermolabile lysin. It was possible to separate the colilysin by high speed centrifugation from the bacteria in all the other substrates used except Robinson's medium, in which the lysin became completely inactivated during centrifugation. Filtration of colilysin was not successful. The colilysin is extremely labile and could not be stabilized by culture of the bacteria in O_2 or N_2 atmospheres or by addition of reducing substances. In inactivated lysin it was not possible to demonstrate the presence of any factor with an inhibitory effect on active colilysin. During the production of colilysin a certain correlation appeared to be present between diminishing hemolytic power and decline of the pH below 6.0. It

was not possible to re-activate inactivated lysin by raising the pH. Inactivation of colilysin thus appears to be an irreversible reaction.

4) Colilysin was precipitated with methyl alcohol at —10° C. and with a saturated solution of $(NH_4)_2\ SO_4$ at ±0° C. Solution of the lysin precipitate was difficult in H_2O, physiological saline solution, and N/10 Sörensen's phosphate buffer at various pH values. Attempts to dialyze colilysin precipitated with $(NH_4)_2\ SO_4$ against H_2O were unsuccessful.

5) However, the lyophilization of colilysin precipitated with methyl alcohol increased the concentration of the colilysin 2- to 3-fold. The yield of active lysin in powdered form was c. 30 per cent of the original amount of active lysin produced in the medium.

6) The hemolytic activity of colilysin in the culture fluid could not be maintained during storage for any great length of time at +4° C. or —18° C. Alcohol-precipitated and lyophilized lysin stored in powdered form in vacuum-sealed ampoules at —18° C. retained its hemolytic power for 8 months. The addition of reducing substances or change of the pH did not improve the stability of the fluid lysin.

7) Anticolilysin was produced in rabbit serum by immunization of the animals with colilysin. In cross titrations of immune serum with homologous and heterologous colilysin, the lysin was found to be independent of the strain which had produced the antigen. When AST and Asta standard sera were titrated with colilysin no inhibition of hemolysis occurred. In cross titrations of 100 different sera with colilysin, streptolysin and staphylolysin there was no significant overlapping with these antihemolysin reactions. Accordingly, anticolilysin and colilysin must be regarded as specific and clearly different from antistreptolysin and streptolysin, and from antistaphylolysin and staphylolysin, respectively.

8) On the basis of the anticolilysin titers obtained for sera from 206 »healthy» subjects, the titers below 400 were regarded as normal, a titer of 400 as moderately elevated, and titers over 400 as elevated (in 8 per cent of »normal» subjects).

9) The standard error of the anticolilysin reaction was ±15 per cent.

10) In a total of 230 patients, anticolilysin titers of 400 or over were seen in 50 per cent or more of the cases in many disease groups, *i. e.:* Pyelocystitis, cholecystitis, cholangitis, cholelithiasis, infec-

tious abortions, and chronic infectious gynecological diseases. Elevated titers were also seen in individual cases in other disease groups.

11) At least in the disease conditions listed under 10), the anticolilysin reaction may probably be accorded a certain degree of clinical importance. In studying the part played by *Esch. coli* in various diseases, a statistical analysis of the results of anticolilysin titrations in a number of cases of the same disease may provide significant information.

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